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SOME BIOLOGICAL IMPLICATIONS OF STUDIES ON INFLUENZA VIRUSES*

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The Walter and Eliza Hall Institute, Melbourne, Australia

I. THE PROCESS OF INFECTION BY THE VIRUS
II. REPRODUCTION AND VARIATION IN INFLUENZA VIRUSES
III. THE ECOLOGICAL APPROACH TO THE COMMON VIRUS DISEASE OF TODAY

LECTURE I

THE PROCESS OF INFECTION BY THE VIRUS

In 1933 I was working at the National Institute of Medical Research in London at a time when virus research was just entering its modern phase. At Hampstead in that year Elford had developed his gradocol membranes, and the first relatively accurate measurements of the size of viruses had been made. Barnard had confirmed these sizes for some of the largest viruses by his method of ultraviolet microphotography. I had become, I think, Goodpasture's first overseas disciple in the use of the chick embryo for the growth of viruses and Smith, Andrews and Laidlaw had just transmitted the virus of influenza to the ferret. It was an exciting time, and it was inevitable that my two years in England then, should have determined my subsequent field of work in Australia. In 1935 I isolated a Melbourne strain of influenza virus and in the subsequent year adapted it to grow freely on the chick embryo. Since then the influenza virus and the chick embryo have held their places at the center of all our activities at the Hall Institute.

Influenza is a disease that once in this century provided the greatest of all epidemics but at the present time is of no very great medical or economic importance. In these lectures I shall be concerned only in passing with the medical and historical aspects of influenza. I want to take the influenza virus simply as a typical virus and discuss its interaction with its hosts from the broadest biological standpoint.

It so happens that there are characteristics of the influenza group of

* The Herter Lectures delivered at the Johns Hopkins Hospital, October 9, 10 and 11, 1950.

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viruses and techniques available for their study which allow more effective experimentation than is possible with any other group of viruses attacking human or animal tissues. Those who work with bacterial viruses, bacteriophages, may claim with justification that theirs is even more tractable material. Bacterial viruses may seem a little remote from the main stream of biology, but it has been very impressive to observe how curiously similar in their behavior are the bacteriophages and the influenza viruses. Living matter is everywhere constructed of the same building stones. To the biochemist there are more resemblances than differences between a bacterial cell and the cell of a tobacco plant or a chick embryo.

If we are in a position to study in detail the interaction between a virus and the cell it infects, we are liable to learn a great deal about both the organisms concerned and much of what we learn may turn out to be relevant in wider fields. My essential theme then is the interaction of parasite and host particularly at the cellular and subcellular levels as exemplified in the influenza virus infections. It is only a tiny corner of biology, but I think that if I wanted a text for this discourse, I should take Hamlet's words "I could be bounded in a nutshell and count myself a king of infinite space. . . ."

There are three aspects of the functioning of influenza viruses with which I wish to deal. Two of them are at the microscopic level: (1) the means by which the virus enters the cell and all the implications of that mechanism, and (2) the process of virus multiplication in the cell and the phenomena of mutation, interference and recombination associated with this. The third at the macroscopic level is concerned with the processes by which the virus survives in nature and incidentally to that survival produces human disease.

HAEMAGGLUTINATION

Any discussion of the means by which the influenza viruses make effective contact with and infect susceptible cells must of necessity begin with the phenomenon of virus haemagglutination. In 1941 Hirst (1) and independently McClelland and Hare (2) noticed that when allantoic fluid from infected chick embryos was contaminated with blood the red cells formed coarse clumps. By following up this observation it was relatively easy to devise techniques by which this haemag-

glutination could be tested quantitatively; and once it had been shown that the activity was a function of the virus particles themselves, used as a relatively accurate means of titrating virus. Once this work of Hirst became generally known the use of allantoic fluid virus and its titration by haemagglutination became the universal practice of laboratories dealing with influenza. A great variety of supplementary techniques have been developed by which various qualities of the viruses can be demonstrated or measured, all using the presence or absence of haemagglutination as the final index.

Quite apart from its value as a basis for characterization and titration of viruses, the phenomenon of haemagglutination in itself has called for a great deal of investigation. Suitable experiments show that when virus and red cells are mixed in the cold most of the virus is rapidly adsorbed to the cells which are at the same time agglutinated. The agglutination is merely an index of the adsorption. Whenever a red cell carrying a number of adsorbed virus particles comes into contact with another, a proportion of the virus particles will have an opportunity to adsorb to both cell surfaces and as it were provide bridges to hold the cells together. In this way clumps rapidly form and settle to the bottom of the tube giving the characteristic pattern of granular sediment.

The further study of the haemagglutination phenomenon was also initiated and developed by Hirst who has been responsible for nearly all the basic discoveries on which the modern interpretation of the reaction is based. Two of these are of particular importance. The first is the sequence of adsorption and elution of virus. If the reaction is carried out at 37°C., three phases can be observed: 1) adsorption of virus to the cells with a precipitate fall in the concentration of free virus, 2) a period of an hour or two in which the cells are unstable and there is little or no free virus, and 3) a phase of elution with increasing concentrations of virus in the fluid and the appearance of stable cells inagglutinable by a fresh sample of the same virus. It is possible to adsorb virus on cells, wash the cells in the cold and then allow elution to occur into a fresh volume of saline. This eluate, containing nearly all the original virus, can be used to initiate a similar cycle; and this can be continued indefinitely with only casual loss of virus in the process. At each stage a large number of red cells are rendered inagglutina-

ble by the virus. Such behavior as Hirst recognized (3) is characteristic of an enzymic reaction.

Hirst's second basic discovery (4) was that the respiratory surface of the ferret lung behaved toward virus just as the surface of the red cell did. This immediately suggested that in some ways at least the red cell reactions could be used as a model for what occurs in the much more important interaction between the virus and the susceptible cell that it will eventually parasitize. In the excised lung the virus is first adsorbed then after a period re-liberated. After the re-liberation the lung is incapable of adsorbing more virus. It should be stressed that this holds only for the excised lung. In the living animal virus is adsorbed but is not re-liberated.

The provisional conclusion to be drawn from these two types of experiments was that adsorption of a virus to a cell surface component, followed by its release through enzymic or pseudoenzymic destruction of that component, was an essential part of the process of infection and therefore merited detailed study. In 1945 the systematic study of this process was begun in the Hall Institute—it would probably be more correct to say that it now became a conscious objective rather than a side-line to studies primarily concerned with the development of methods of immunization.

With Hirst's hypothesis that the virus carried an enzyme whose substrate was the receptor of the red cell surface as a starting point, there were several lines of approach to the main problem of defining the chemical nature of the substrate; and the change induced in it as a result of the enzyme action. The most direct approach would have been to try to isolate the substrate from large amount of red cells—a task quite beyond our physical equipment. A less direct but more practicable approach was to look for soluble enzymes which might have the same easily detectable effect on the red cell surface as the virus. We therefore tested known enzymes, tissue extracts, bacterial culture filtrates, and so on to determine whether any of these could modify red cells as the influenza viruses modified them. The test was simply to expose washed red cells to the action of these enzyme preparations for as long a time as was consistent with the cells remaining intact. The cells were then washed and tested to see whether they were agglutinated by a standard influenza virus. The only hopeful material found in the

first survey was the toxin of *Cl. welchii*, Type A, which had a well marked effect of this character and which was extensively investigated from this point of view by McCrea (5).

Welch toxins are notoriously complex and are also highly haemolytic and a much more suitable source of an enzyme that would do essentially what the viruses did was found in the cholera vibrio. The reason for our examining the cholera vibrio may be of some interest. After one allows a virus to act on red cells for several hours, the cells may cease to be agglutinated and form stable suspensions. It was of some importance to determine whether such cells were inagglutinable by all influenza viruses or by some only, and for tests of this sort one naturally wanted cells acted on and stabilized by all the viruses under study. Some of these however consistently failed to yield stable cells. To obtain workable material these unstable cells were treated with an antiserum to the virus. This removed the virus and under proper conditions gave stable cells. But if too large an amount of immune serum was used, the cells were even more firmly agglutinated. Investigation of this point showed that when cells were acted upon and stabilized by any of the influenza viruses, they became susceptible to agglutination by *any* serum up to a serum dilution of 80 or thereabouts. Only when the immune serum contained enough specific antibody to be effective at a dilution of some hundreds was it satisfactory as a stabilizing agent.

The agglutinating action of *any* serum called to mind the Thomsen-Friedenrich phenomenon of panagglutinability. In 1926 they found that human red cells used for blood grouping were sometimes agglutinable by any serum—not only those whose activity was appropriate to the type of blood cell in the particular sample. This change was traced to the action of bacterial contamination on the blood samples and by Friedenrich (6) to a soluble bacterial enzyme. He found that an enzyme with this property of producing panagglutinability was produced in cultures of certain diphtheroids and vibrios. It became of immediate interest therefore to see whether bacteria of this sort would produce changes in red cells at all similar to those induced by the influenza viruses. The only readily available cultures of types used by Friedenrich were of the cholera vibrio. The first test with a vibrio filtrate showed at once that not only did it produce panagglutinability of human or chicken red cells, but it also rendered them inagglutinable

by influenza viruses (7). Since then what we have called the receptor destroying enzyme of the cholera vibrio, RDE, has been a reagent for which there has been a steadily increasing demand in my laboratory —and which has become of considerable interest to a number of American and European workers.

THE PROPERTIES OF RDE

During the last year Ada (8) has made active progress toward purification of the enzyme using successively methanol precipitation, adsorption to and elution from red cells, and ammonium sulphate precipitation. The fact that the enzyme could be adsorbed to red cells in the cold at pH 9 and after the cells had been washed, eluted into a smaller volume of saline provided a relatively specific biological means for its purification. Subject to the qualification that the work has not been done with physically pure enzyme, I believe that all the activities which I shall describe as being manifested by preparations of RDE, are due to a single enzyme. I shall say something about its chemical activity, but for the most part I shall be concerned with its relation to the influenza viruses. From these relations arises our current hypothesis that the *cholera vibrio secretes a soluble enzyme which in all essentials is identical with an enzyme built into the surface structure of the virus particle.*

It is advisable first to state the conditions under which the enzyme functions. It is completely inactive in the absence of Ca, and its action can be stopped immediately by the addition of a Ca-deionizing agent like citrate or sodium hexametaphosphate. It is active over a fairly wide range of pH with an optimum at pH 6.2. The enzyme is destroyed by 30 minutes heating to 55°C. in the form of crude filtrate; but if an excess of Ca is added and the pH adjusted to 6.0, such heating has no effect. The enzyme is a rather poor antigen but by the use of adjuvants or of large immunizing doses antibody capable of specifically inactivating the enzyme can be obtained. A unit of RDE is defined as the amount capable of just destroying the agglutinability of 0.25 cc of 1 per cent fowl red cells by Melbourne influenza virus under standard conditions. An average crude filtrate can modify 10–20 times its own volume of packed red cells in 30 minutes at 37°C. Present indications are that the content of actual enzyme in such a preparation is of the

order of 0.005 per cent so that a 1 per cent solution would modify some 2000 times its volume of cells. The enzyme then is defined and measured by its action on red cells so that it is appropriate to deal first with its action on these cells.

The most illuminating way to demonstrate the action of RDE on red cells is to make a set of serial dilutions of the enzyme and allow these to act on cells for 30 minutes at 37°C. The action is then stopped by addition of citrate and the cells washed well in citrate saline. If these washed cells are made up to 1 per cent and tested for susceptibility to agglutination by different viruses, the viruses will be found to arrange themselves in a characteristic linear order which we have referred to as the receptor gradient. With each increase in the action of RDE the cells become insusceptible to the action of an increasing number of viruses. The order in which susceptibility to agglutination by the different viruses disappears is approximately but not exactly the same as that found when virus action is used to remove receptors. In our work using allantoic fluid virus without concentration to act on the red cells we found in general that as soon as a cell suspension ceased to absorb or be agglutinated by virus, then the enzymatic action of the virus ceased. For each virus there was a fairly sharp level beyond which receptor destruction did not proceed. In carrying out similar experiments Hirst used virus which had been concentrated 10-fold by centrifugation and obtained different results. He confirmed that there were well marked differences in the speed with which viruses destroyed agglutinability of cells by the viruses of the gradient series, but he found that under his conditions nearly all the viruses could eventually remove agglutinability by any virus of the series. The discrepancy is not a serious one and will probably be almost completely resolved by more detailed studies of virus-cell interactions soon to be published by Ada and Stone (9).

Protection against experimental infection with RDE

So far we have been concerned only with the action of RDE on the red cell. It was clearly likely from Hirst's experiments that pretreatment of the excised ferret or mouse lung with RDE would render it incapable of adsorbing virus drawn into the lung substance through the trachea. This was shown to be the case by Fazekas (10) who, using

the mouse lung preparation, was able to duplicate in detail almost all the reactions shown by red cells in test tube experiments.

The next step was to pass to *in vivo* experiments designed to test the hypothesis that union of virus with receptor was necessary for infection to occur. If this is so, then infection should be prevented by removing the specific "receptors" from the cell surface. Stone (11) has been able to show that this is the case in the two favourite experimental sites for studies on influenza virus infection—the allantoic cavity of the chick embryo and the mouse lung.

In both situations the effectiveness of the preventive action depended on the strain of virus being tested. In the mouse lung tests, the order was very close to the order of viruses in the receptor gradient as tested on red cells. Very limited action of RDE for instance will render cells inagglutinable by strain MEL while prolonged action is needed to remove reactivity with Swine. Similarly, as can be seen from Stone's figure, RDE *in vivo* protects well against MEL but hardly at all against SW. Against the more susceptible viruses the effect of mixing the inoculum with high titre RDE (1500 U per dose) resulted in about 100–1000-fold reduction in the intensity of its action.

In the chick embryo there were again some viruses highly susceptible and others much less so to the effect of RDE on the receptive surface of the allantois. Here the difference did not relate so much to the position in the receptor gradient as to the degree to which the strain had been adapted to passage in the allantoic cavity. Recently isolated strains had much more difficulty in overcoming the RDE effect than old well-established laboratory strains.

Various other aspects of the phenomena will be found discussed in Stone's papers. A wide variety of control experiments were included, and there can be no doubt about the reality of the protective effect or that it is actually due to the receptor destroying enzyme.

The nature of the substrate of virus enzyme and RDE

If an important aspect of influenza virus activity is due to an enzyme which forms part of its surface structure, it is obviously necessary to take steps to determine the substrate of the enzyme and the chemical change of the substrate produced by its action.

The first lead to a chemical attack on the substrate of RDE and the

influenza viruses came from Francis' (12) observation that normal human and animal sera showed a high power to inhibit haemagglutination by the influenza B virus LEE provided the virus had been heated to 56°C. for 30 minutes. This inhibition was quite independent of the presence or absence of specific anti-influenzal antibody in the serum.

In the course of work on this phenomenon Anderson (13) attempted to absorb out the inhibitor with cells that had been treated with RDE. He found variable results which on analysis showed that while the treated cells themselves had no action, any contaminating RDE had a rapidly destructive effect on the inhibitor in the serum. Following this finding he showed that influenza virus B when incubated with serum destroyed what we called the Francis inhibitor. At about the same period de Burgh et al. (14) were isolating a complex polysaccharide from human red cells and showed that the inhibitory action of this receptor substance was also destroyed by virus acting enzymatically.

It thus became clear that there existed at least one soluble substrate for virus enzyme and RDE in normal mammalian serum, and that we had available a general method for recognizing such substrate material. It would inhibit agglutination by heated LEE virus but not by unheated virus, and this inhibitory action could be rapidly destroyed by RDE. Leaving aside for the moment the problem of why heated virus was inhibited and not active virus, work was concentrated on finding and as far as possible defining the chemical character of substrate materials. In addition to serum many tissue extracts and secretions were found to contain Francis inhibitor. All had in common mucopolysaccharide material which from a general accumulation of evidence rather than from any single crucial point was gradually recognized as being or containing the essential substrate. A wide variety of secretions containing glandular mucins were highly active, e.g. human saliva, gastric mucus, bronchial mucus and cervical (uterine) mucus; the inhibitory titre of these materials was roughly proportional to their viscosity. Egg white was a very active inhibitor. Purified blood group substance of human (ovarian cyst) origin was active to high dilution but similar material of swine origin was quite inert. Synovial fluid and purified hyaluronic acid were not inhibitory. Finally, as already mentioned, de Burgh et al. (14) isolated from human red cells small

amounts of inhibitory material of polysaccharide character which in our hands showed the same concentration of activity on heated virus (as compared with its action on active virus) and susceptibility to RDE.

In addition to its substrate the specification of an enzyme requires a statement of the chemical change induced in the substrate by the enzyme's action. In the present instance the substrate was clearly a macromolecular substance from which enzymic action might, by analogy with other enzymes known to attack polysaccharides, be expected to split off smaller dialysable molecules. An investigation to test this probability was initiated by Gottschalk and Lind (15). The most convenient source of crude substrate was egg white. They first showed that the active component was contained in the fraction (ovomucin or ovo-mucoid B) which is insoluble in distilled water. A considerable concentration was effected by simple precipitation and washing with distilled water followed by resolution in salt solution. This is very far from pure ovomucin and especially contains large amount of lysozyme, but it provided satisfactory material for their experiments. The semipurified ovomucin was dialysed against salt solution for use as substrate, and virus concentrated by elution from red cells was also freed from any dialysable material to serve as the enzyme component. The materials were then mixed and allowed to react until 90 per cent of the inhibitory action of the mucin had been lost. The mixture was dialysed for 48 hours against a small volume of saline into which any dialysable products of the reaction could pass. Concentration of the dialysate showed a product not present in any of the appropriate controls. It contained nitrogenous material which on mild alkaline hydrolysis showed reducing sugar and amino-acid. In all essentials the behaviour of the dialysable split product could be duplicated with the N-glycoside of lysine or other amino-acids, the chief features being production of humin substances on acid hydrolysis and a characteristic purple colour with *p*-dimethylaminobenzaldehyde.

Preliminary work has shown similar products from the action of RDE on ovomucin and of influenza virus on human blood group substance A. This general line of work is being actively followed by Gottschalk, and I have little doubt that a much clearer statement of the chemical nature of the action will soon be possible.

Before leaving the question of the virus-enzyme substrate, brief mention should be made of Whitten's (16) finding that gonadotrophin is rendered biologically inert by the action of either RDE or active influenza virus. This holds for three different forms of the hormone prepared from pregnancy urine, from pregnant mare serum and from pituitary substance and is effective *in vivo* as well as *in vitro*. The experiments were initiated because of the known mucoprotein nature of gonadotrophin and would seem to indicate that the particular aspect of the molecule attacked by the enzyme is of high significance for its hormonal activity.

THE NATURE OF THE RED CELL SURFACE

The red cell has been used so extensively in general biology as an indication of the haemolytic and agglutinating action of a wide variety of substances that it is fair to say that the nature of its cell surface presents a classical problem. The action of RDE and the viruses of the influenza group on the red cell has provided some new angles of approach to that problem. I have already indicated some of the conclusions that we have arrived at, but I have said nothing about a number of other points of interest.

1. Mumps and Newcastle disease viruses differ from the influenza viruses in being haemolytic for all the types of red cell that they agglutinate. Newcastle disease virus is the more active and is the only one with which we have worked. By partial purification with methanol in the cold the intensity of haemolysis is greatly enhanced. The unique feature of viral haemolysis is that it requires the adsorption of the virus by the same receptors as are concerned with haemagglutination. RDE is quite non-haemolytic; but after its action on cells, they are insusceptible to haemolysis by NDV. It can be shown that NDV itself can protect cells against its own haemolytic action. Every experiment is essentially a race between the normal adsorption, elution and destruction of receptors on the one hand and haemolysis on the other. As soon as receptor destruction is complete haemolysis ceases.

In some way closely associated with haemolysis is another character in which NDV differs from the influenza viruses. This is its capacity to make a firm irreversible union with the cell surface which cannot be dissociated with either RDE or immune serum. Neither process

can occur with cells treated with RDE nor at temperatures below 20°C. Burnet (17).

2. When red cells are treated with viruses, their electrophoretic velocity is reduced, Hanig (18); and this reduction is taken much further by RDE. Under standard conditions the value for the human red cell is very constant at 1.3 μ/sec/volt/cm. The full action of RDE reduces this to 0.17. In other words virtually the whole negative charge of the red cell at pH 7.35 is due to components which are removed or neutralized by the action of RDE. Ada and Stone (9) have recently completed an elaborate analysis of the changes produced in human red cells by viruses and RDE used singly and in succession, the change in electrophoretic motility being correlated with the loss of agglutinability by different viruses of the receptor gradient. The results indicate a very complex but consistent pattern of receptor structure over the cell surface which requires the postulation of three "sequences" of action. This may mean that in addition to simple destruction of receptors there may be two secondary results of enzymic action—one haemolysis, the second concerned with reduction in electrophoretic motility without change in agglutinability by viruses.

This very striking physical change produced by RDE is a challenge to the physical chemist, and my colleagues are working on the possibility that PO_4^{III} groups are removed in some secondary reaction. No evidence has however yet been obtained to substantiate the idea.

3. If RDE, concentrated and freed of toxic substances present in the original filtrate, is injected intracardially in a guinea pig in adequate dose, all the circualting red cells are modified. Their electrophoretic motility is reduced to 0.30, and they are inagglutinable by all but the final viruses in the gradient series. If during the subsequent 3 weeks blood samples of the animal are examined, there is progressive evidence of "repair"; and the cells are approximately normal in 20 days. This takes place without evidence of any abnormal blood destruction or of any increase in reticulocytes to suggest unduly active replacement. Here then, by an entirely different technique, is further evidence that the red cell membrane, unlike the contained haemoglobin, is a metabolically active living tissue.

4. Red cells treated with RDE are serologically altered. They are agglutinated by a component, T-agglutinin, of all normal human sera

except those of infants, which has no action on normal cells. In addition a new immunologically distinct component appears which can be recognized by appropriate immunization of rabbits and cross-absorption experiments with their sera Burnet and Anderson, (19).

The whole series of finding impresses me as adding an important new set of technical approaches to the study of the red cell surface and to a considerable extent of the surface of the vertebrate cell in general.

THE "INDICATOR STATE" OF INFLUENZA VIRUSES

It has already been stressed that the "Francis inhibitor" present in normal serum and the similar inhibitors of egg white and other mucinous materials are active only in inhibiting haemagglutination by *heated* influenza B virus. They do not inhibit the action of active virus on red cells and do not diminish its infectivity. It was first noted by Briody (20) that influenza B virus heated to *55° C.* failed to elute from agglutinated red cells. Soon afterwards when the capacity of active virus to destroy the haemagglutinin inhibitors in mucoid solutions had been recognized, it was shown that any treatment which destroyed the enzymic activity of the virus on mucoid inhibitor while leaving its haemagglutinating power intact, gave a preparation sensitive to inhibition Burnet (21). Hirst (22) made similar observations using both heat and periodic acid to induce the change in the virus. In both laboratories the term "indicator state" is used to characterize virus preparations whose haemagglutinating action is inhibited by dilute solutions of typical inhibitors such as those in egg white.

The conditions needed to induce the change from the active form differ widely with different strains. Some strains which are only weakly active as enzymes are significantly inhibited in the unheated state and require only very minor degrees of heating for conversion to the indicator state. With our strain of PR8 Stone (23) was able to show that a reversible change could be induced by treatment with citrate. When converted to indicator the virus had no enzymic action. On reversing by use of calcium, enzymic activity returned and concomitantly the virus was no longer in the indicator state. A number of influenza A strains were not convertible by heat alone but became excellent indicators of inhibition when heated in the presence of citrate at pH 8.5.

The picture emerging is broadly that when we have the three component system—the virus (as enzyme), the soluble mucoid and the red cell (as two competing substrates)—a temporary equilibrium can be reached which with active virus finds most of the virus on the surface of the cells. When enzymically inactive virus is used, adsorption to the first substrate presented is not readily broken down; and in the ordinary experimental set up this is manifested as a high inhibitory titre.

A complementary phenomenon can be shown by the use of very small concentrations of periodate on either the cellular or the soluble substrate. Fazekas (23*a*) found that at a certain level of periodate treatment red cells had the normal capacity to bind virus and be agglutinated but no subsequent elution of virus occurred. Similarly soluble mucoids appropriately treated with periodate were capable of inhibiting not only virus in the indicator state but also active virus Burnet (24). Here we have a phenomenon analagous to the well known competitive inhibition of enzymes by compounds structurally related to the substrate.

Very full accounts of these phenomena will be found in papers from the Hall Institute, and all the essential findings have also been demonstrated in Hirst's laboratory. The interpretation is, however, by no means clear. Even within my own laboratory there are different points of view. I have felt that it is necessary to ascribe all the phenomena which depend on the presence of cell receptors—i.e. which do not occur after a cell has been treated with RDE—as being manifestations of specific enzyme-substrate interaction. If this is true, then we are obliged to envisage a wider variety of such interactions than have yet been accepted for classical enzymes. The concept of competitive inhibition is well established, but as far as I know no one has found it necessary to postulate a change in an enzyme by which it retains its specific adsorption to the substrate but is incapable of inducing chemical change. In most systems however it would require elaborate experimentation to detect inactive enzyme retaining power to adsorb the substrate and differentiate an effect due to this from other types of change taking place in the course of the reaction.

In another lecture (Burnet, 25) I have suggested that in these interactions of an enzyme embedded in the virus particle surface with its

substrate, there are very great opportunities for the enzyme chemist. Once the chemical nature of the action of RDE is adequately defined—and I am very optimistic that this can be done within the next two years—the red cell, virus, soluble mucoid substrate and RDE system should offer great dividends to a thoroughgoing investigation. I know of no other set up in which enzyme on a particulate carrier can react with a living cell structure to give a variety of easily measurable effects—the cell concerned being the most uniform and readily available of all mammalian cells. In view of the current importance ascribed to particular concentrations of enzymes in the cell, I feel certain that the findings from such an investigation would provide a series of concepts which would help to bridge the gap between enzyme action as it occurs in simple solution and the elaborate series of enzymic reactions which must be concerned in the synthetic processes of the cell.

One further instance may be given of how fruitful in associations with other biological phenomena is this particular field. If one treats ovomucin with periodate, it becomes capable of inhibiting haemagglutination by Newcastle disease virus and two influenza strains; on increasing the amount, the influenza virus inhibitors are lost leaving only the capacity to inhibit Newcastle disease virus. Such a preparation has *all* the qualities of a rather weak Newcastle immune serum including the capacity to inactivate the infectivity of the virus. Here perhaps is a suggestion that antigen-antibody reaction has very much the same character as an enzyme-substrate reaction. It is a suggestion that has often been made before but never with quite such a pertinent example.

CONCLUSION

In this lecture I have tried to do two different things. The first is to indicate what we know about the process by which the viruses of the influenza group make contact with and enter the cells they infect. The second is to convey to others what a productive field of research in general biology these investigations have opened up. In this closing section I should like to say something more about each of these aims.

The picture we have reached of the virus-red cell reaction may be sketched somewhat as follows: Incorporated in the virus surface there are enzymically active groups functionally equivalent to RDE. The re-

mainder of the surface is inactive, but atomic groupings adjacent to the enzyme groupings may interfere with the readiness with which the latter make contact with the substrate on the cell. The varying extent of this interference is responsible for the receptor gradient. On the red cell we can picture a mesh of polysaccharide chains intimately linked with lipid and protein components of the surface. Some of these chains may be in the form of loose filaments with considerable mobility. At numerous points in the meshwork there are what I have called substrate groupings. These may eventually be found to be the points of attachment between polysaccharide and protein, but all that need be assumed is that they are numerous and are removed in some sort of sequence by the enzyme. Their removal is associated with the physical change responsible for the diminished electrophoretic motility of the cell probably by some secondary process rather than directly.

When an active virus particle is adsorbed to the cell we can picture adsorptive unions of varying strength between enzyme groups and substrate nodes. As each node is loosed by enzyme action there is opportunity for another to be engaged. We have been in the habit in fact of speaking of the virus particles as browsing over the cell surface.

When we move to the susceptible cell, the picture is virtually the same except that we postulate that instead of browsing over the surface the virus particle, once adsorbed, sinks into the cytoplasm of the cell by a process Fazekas has called viropexis. Like Hirst, I have felt that the enzymic activity of the virus is so marked that it might be expected to play some necessary part in cell entry; but I think Fazekas is correct in claiming that adsorption only is necessary. The most cogent evidence is the direct demonstration by Isaacs and Edney (26) that indicator virus with no enzymic activity is absorbed from the allantoic cavity, becomes undemonstrable in the cells and is an effective interfering agent.

One possible function for the enzyme which requires further study is that it may be a means of overcoming the protection that the thin film of mucus affords to the cells of the respiratory epithelium over which it lies. Respiratory mucus is a potent inhibitor of haemagglutination and deserves wider study from this point of view than it has received.

The more general aspects of this discussion can be regarded simply

as a plea for the more intensive investigation of the mucopolysaccharides and mucoproteins of glandular type. Just as the recognition of hyaluronidases opened up a wide and productive field of study in the tissue mucins, so I am hopeful that the discovery of at least two mucinolytic enzymes of the cholera vibrio and the renewed interest in lysozyme will stimulate biochemists and cytologists to extend their interest in this field beyond the blood group substances.

RDE can make red cells inagglutinable by influenza viruses and temporarily prevent infection of susceptible cells. It reduces the electrophoretic mobility of red cells to near zero both *in vitro* and *in vivo*. It destroys the capacity of a wide variety of mucoids to inhibit virus haemagglutination. Given intravenously in a rabbit it eliminates the animal's response to the pyrogenetic action of influenza viruses. RDE rapidly destroys the biological activity of gonadotrophic hormone both *in vitro* and *in vivo*. Finally, it has been shown in two laboratories to render mice relatively insusceptible to one of the mouse encephalitis viruses, Columbia-SK.

This surely is enough to indicate that it is a biological reagent of very considerable power and that the mucopolysaccharides and mucoproteins are much more important biologically than they have usually been considered in the past.

I should stress again that the enzymic action of viruses on the red cell is at present a unique example of interactions between enzyme and substrate where both are surface components of relatively massive carriers. I have already indicated that this system is a very convenient one to study and that it presents phenomena which seem likely to provide ideas that may be highly relevant to the functioning of enzymes incorporated in intracellular granules.

As I said at the beginning, this lecture has been in no way concerned with the influenza virus as an agent of human disease. Perhaps the best way of characterizing the approach I have adopted is to look upon the virus as a probe by which we may reach a deeper understanding of cellular structure and function. I hope that I have been able to show that when Hirst demonstrated that the influenza virus was adsorbed to and agglutinated red cells, he opened up a new and fruitful approach to the problems of the cell surface and that the influence of his discovery may extend over a much wider biological field. In the next

lecture I shall be concerned to show that the use of virus as a cellular probe is not limited to the surface but has even more promise as a means of exploring the intracellular environment.

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LECTURE II

REPRODUCTION AND VARIATION IN INFLUENZA VIRUSES

The greatest outbreak of infectious disease in the 20th century was the 1918–19 influenza pandemic. At this distance we do not even know for certain that the microorganism responsible was of the type we now call influenza viruses. From all the indirect evidence, however, I feel no real doubt that if any contemporary bacteriologist had used present

day techniques, he would have isolated strains of virus conforming to the general character of influenza viruses. I should guess, too, that they could have been defined as influenza A strains. The only explanation that can be provided for the lethality of this epidemic which produced 25 million deaths is that the virus strains concerned differed from those from which they were descended as well as from those to which they subsequently gave origin. A series of genetic changes, mutations or recombinations, involving characters concerned with virulence, took place during the pandemic period. Many other conditions may have been involved in determining various qualities of the pandemic of 1918–19, and I shall leave their discussion to the last lecture. Here it is necessary only to point out the immense potential importance of variation in viruses pathogenic for man.

With one side of my mind I can accept the principle that scientific research is justified socially only by its direct or potential contribution to human well-being. From this angle I should define the essential objective of influenza virus research as the understanding of the conditions responsible for pandemic influenza of 1918 type—and the establishment of the conditions necessary to prevent its reappearance. One must regard all the work that has been carried out since 1933 as merely the development of the background of technique and interpretation so as to be able to study effectively any new pandemic that may arise. Since science is concerned only with the reproducible and the recurring and is essentially helpless when confronted with the unique, we are not likely to succeed in our objective of prevention till at least one and possibly more pandemics have been studied by modern techniques. And, unless all our current experience is wrong, the answer is most likely to be drawn from an understanding of the processes and limitations of variation in influenza virus.

In the last few months before my leaving Australia two groups in the Hall Institute, Miss Lind and myself, Dr. Isaacs and Miss Edney, were very intensively concerned with an attempt to develop a genetic approach to the problems of virus variation. I shall therefore make use of a good deal of unpublished material in this lecture.*

* Papers by F. M. Burnet & P. E. Lind accepted for publication by Journal of General Microbiology, 1951.

THE METHOD OF LIMIT DILUTION PASSAGE

With a well adapted strain of any of the influenza-mumps group of viruses a sharp endpoint is obtained in a titration by allantoic inoculation in chick embryos. In our technique we inoculate a volume of 0.05 ml. and incubate for 44 hours at 35°C. It will be usual to find all eggs positive for haemagglutination to a dilution of 10^{-7}, then perhaps $\frac{5}{6}$ at 10^{-8}, and $\frac{1}{6}$ at 10^{-9}. Usually there is no change on a further 24 hours incubation, but with material subject to any manipulation some increase in the number of positives may be noted. Taking the 3 day readings then as final, we find at the limiting dilutions that an all or nothing position holds. Either the fluids contain a high titre of haemagglutinin or they are negative. Those agglutinating red cells have all the other activities of the virus—those failing to agglutinate have none. As far as I know no one has made a rigid statistical study of allantoic titrations to establish formally what I shall now assume, viz. that infection can be induced by a single virus particle and that the proportion of positive eggs in relation to concentration of inoculum conforms to the normal Poisson distribution. If this is so, it follows that when a given dilution gives more negative eggs than positive, the infectious fluids can be regarded as being composed of pure clones derived from a single particle—with about the same degree of certainty as applies to a single colony from an ordinary bacteriological plating.

It seems to me self-evident that just as work on bacterial variation is always based on the character of cultures derived from single well isolated colonies, any work on influenza virus variation must be concerned with "pure clone fluids." The same principle could also be followed with any other virus in which a clear all or nothing endpoint can be obtained.

Our interest in this particular line of approach goes back to the work done by Miss Bull and myself in 1942 (1) on the initial stages of adaptation of influenza A viruses to growth in chick embryos—what we called the O-D change. It is now generally accepted that when an influenza virus is first isolated from a human throat washing: (A) the amniotic cavity is vastly more susceptible to infection than the allantoic sac, and (B) the primarily infected amniotic fluid or embryo lung emulsion agglutinates guinea pig or human red cells to a titre corresponding to

the virus concentration but does not agglutinate fowl cells even in the cold. These points have been confirmed both in America and England. What has not been confirmed outside of our laboratory is that it is possible to maintain these two characters, of inability to grow in the allantoic cavity or to agglutinate fowl cells, by passage in the amniotic cavity at limiting infective dilution.

Nevertheless we have found it possible to retain the strain BEL isolated in 1942 in the original O phase for 24 passages, to recover it as required from material frozen in dry ice and to continue such passage up to the present—eight years since isolation.

The situation in regard to the O-D phase variation is, I think, of crucial importance in regard to any genetic approach to the influenza viruses. In the first place I have felt ever since 1942 that nearly all the work that has been done in the laboratory with the influenza A viruses has been done with other things than the human pathogen. Influenza A virus, the human pathogen, does not multiply in the allantoic cavity, does not produce lung lesions in mice and does not agglutinate chicken red cells. This difference may be as unimportant as all influenza virus workers including my own group tend to believe or at least to act as if they believed—but its existence should always be kept in mind.

Secondly, the adaptation of a human influenza A virus to typical growth in the allantoic cavity and to mouse pathogenicity is a striking example of virus adaptation to an experimental host, but essentially it resembles what happens whenever any virus is transferred from one host to another. The fact that a sharp change, easily demonstrable in the test tube, occurs on adaptation merely makes the example particularly amenable to experimentation.

Since 1942 we have made several re-examinations of the O-D phenomenon as further knowledge developed and the account I shall give differs somewhat from that of our 1943 paper—mainly because at that time we had failed to grasp the significance of some of the intermediate phase fluids that we obtained.

From the fact that the full susceptibility of the chick embryo to human A virus does not develop till the 13th day of incubation when the parabronchi of the embryo lung commence to bud out to what will become the respiratory capillaries, we are still inclined to regard the

susceptible element after amniotic inoculation as the developing pulmonary epithelium. Human virus particles can enter such cells, multiply freely and cause gross necrosis with reliberation of virus. This virus accumulates in the amniotic fluid and, in the earlier stages only, in the lung. The amniotic fluid as harvested or a saline emulsion of the lung spun clear of cells then represents a population of virus particles all derived from a small number of particles of human virus present in the inoculum. The dominant form is still that which was present in the inoculum and the fluids show no chicken cell agglutination.

If such infected amniotic fluid or lung emulsion is transferred at low dilution to another embryo by the same route, the resulting infection will give a fluid capable of agglutinating fowl cells sometimes almost to the same titre as guinea pig or human cells—more often considerably less. A change in the character of the fluid, as determined by the average character of the virus population it contains, has taken place. This change will be accentuated by a further series of passages, and usually within 2 or 3 the full D character of laboratory strains is evident.

Theoretically this change in character might have 1 of 3 different explanations: (A) The inoculum from the human throat may have contained a mixture of genetically distinct virus strains, and one of these initially in small amount has been favoured by the conditions in the embryo and has become the dominant form; (B) By virtue of a process analogous to the development of adaptive enzymes in bacteria as pictured by Hinshelwood, growth in chick embryo cells has resulted in a gradual adaptation of the enzymic-adsorptive pattern of the virus to allow its more effective growth in the new host and incidentally allowing it to become capable of agglutinating fowl cells; (C) Random mutations are constantly occurring; any mutant better adapted to infect and multiply in the new host cells will increase at the expense of the original form; and, by successive mutation, a marked change in the character of the virus can result.

There is one way in which a choice can be made between these alternatives. This is by the method of successive passage at limiting dilutions. The O character of an amniotic fluid means only that the dominant form in the fluid, if present alone, would give similar reactions. There may well be a considerable minority of particles which, if *they*

were present alone, would provide a fluid with quite different properties. Now if we dilute the fluid to its 50 per cent infective level so that on the average less than 1 infective particle is present in the standard inoculum, we can inoculate a series of eggs with the certainty that a majority of the infections produced result from the descendants of a single particle—i. e. the fluids represent what we may call a pure clone of virus. By studying the quality of fluids obtained at successive limit dilutions, in contrast to those obtained by successive passage at low dilutions, we can clearly distinguish between the alternatives.

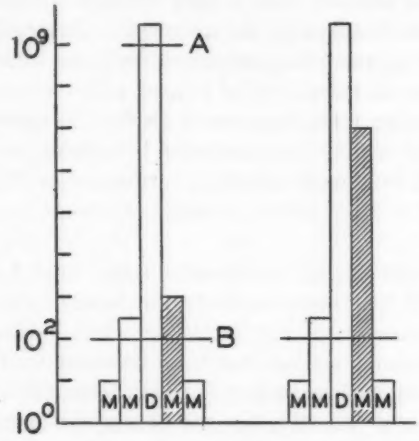

FIG. 1. A diagram to illustrate the presumed composition of virus populations in an infected fluid from the chick embryo. Each column represents the number of virus particles of a given genetic type (D, the dominant type corresponding in the first figure to the type inoculated, M. mutant types. The shaded column denotes a mutant capable of growing more readily in the embryo than the original.) If transfer is made by limit dilution (line A), successive populations all have the composition shown in the first figure. When transferred at a dilution of 10^{-2} (line B), the resulting population will have the composition shown in the second figure and on further transfer the shaded column will become a new dominant form.

On (A) the fully adapted form would appear immediately and remain constantly present.

On (B) each passage would show a further stage of adaptation. Since the number of generations needed to reach standard titre fluids

would be slightly greater with limit than with low dilutions, the change should be slightly more evident with the former.

On (C) limit dilution passage should retain the character of the original indefinitely provided that no mutants arise with an extreme advantage over the original in multiplication rate.

Experimentally the 3rd alternative is found to hold with the reservation that at times a mutant will appear early and overgrow the original form. So far we have never failed on repeating that passage to obtain the O type.

Without going into the experimental detail on which the interpretation is based, our present conception of the O-D phase change in influenza virus is as follows:

The true O form has some special survival advantage as a human pathogen; and except in instances where very large amounts of virus are present in the throat washings, only O phase particles are inoculated into the amniotic cavity with the primary inoculum. The O phase particles are characterized by their capacity to be adsorbed to human, guinea pig and pigeon erythrocytes presumably because of some specific configurational relation to the receptor mucopolysaccharides concerned. There is evidence that a typical avian mucoid (ovomucin from egg white) is not an inhibitor for the indicator form of O nor a substrate for the active form while a typical human mucoid from ovarian cysts does so serve in both capacities. Probably to a significant but limited extent the mucoid patterns on the red cells have some features of molecular pattern in common with those of potentially susceptible cell surfaces and of the mucinous secretions in the same species.

When a population of descendant virus particles builds up, mutational changes in the virus configurations responsible for adsorption will occur in random directions. The first detectable change is one by which adsorption to the fowl red cell becomes possible in the cold (4°C.) but not at room temperature. This is shown by the appearance of a weak agglutination at 4°C. which disappears if the racks are brought to room temperature and *does not reappear when the cells are again allowed to settle in the cold.* When an egg fluid is obtained in which there are majority of particles at this particular stage, one can demonstrate very readily the so-called *in vitro* transformation of O into D.

One does a normal titration with fowl cells, and when the cells have completely settled at room temperature, there is no more than an equivocal roughness around the edge of the cell deposit in some of the early tubes. If one shakes and allows the cells to settle again in order to make sure, the negative result is quite unequivocal. Now if the same virus is heated to a temperature between 50–55°C., it will be found to agglutinate fowl cells quite actively—not producing quite as firm a "shield" as an adapted strain—but unmistakably agglutinating. Similarly if one treats the fowl cells with an appropriate concentration of periodate, the untreated virus is apparently transformed into the D type.

The explanation is simply that weak adsorption to fowl cells occurs, and at moderate temperatures this is rapidly followed by enzymic action and elution of the virus. If this process is completed in 60 or 80 minutes at room temperature, the titrations will appear negative. However, partial agglutination will nearly always be seen if the tubes are scrutinized carefully during the earlier stages of the process at room temperature and will be quite apparent at 0°C. Any means by which the enzymic action and elution of the virus is prevented will result in persisting visible agglutination—it is immaterial whether this is attained by destruction or damage to the virus enzyme on the one hand or by conversion of the substrate to insusceptible form by the action of periodate. I must stress that with "pure O" virus none of the methods of transforming "O into D *in vitro*" mentioned by various authors are effective. Our primary criterion is absence of any agglutination of fowl cells at 0–4°C.

Further adaptation develops as a result of step mutations by each of which a more effective adsorption to cell receptors or to similar soluble mucoids, and as a corollary a more active enzymic action can take place. It is, of course, highly probable that in the course of adaptation to a new host other characters than those concerned with adsorption and enzymic action will be modified; but these so far are the only ones we have studied in detail.

This may appear to be overstressing a relatively trivial matter, but I have felt that we have never quite succeeded in impressing other workers with the reality of the O-D phase change or with the concep-

tion that in a highly mutable agent like influenza virus *the original character can usually be maintained in a new host provided passage is carried on at limiting or near limiting dilutions*. This principle is entirely analogous to the normal bacteriological practice of avoiding degeneration of any bacterial strain under constant subculture, by frequent replating and choice of typical colonies.

Another example of the same principle may be used as an introduction to the second main theme of this lecture which is concerned with the possibility of transferring a quality from one strain of virus to another—the possibility of genetic recombination. This work has all

TABLE 1

Comparison of Two Strains Derived from Influenza A Virus WS

	NEURO-WS	WSM
Pathogenicity		
Mouse I. C.	++	−
Mouse I. N.	++	++
Chick embryo C. A.	++	+
Chick embryo I. V.	+ *Tissue haemorrhage*	− or delayed +
Enzymic action	Trace	Normal
Conversion to indicator	*Fails by all methods*	52° for 30′
Thermostability of		
Haemagglutinin	54° for 30′	66° for 30′
Infectivity	54° for 30′	58° for 30′
Position in receptor gradient	Earlier	Later

been based on the use of the neurotropic strain of WS produced by Stuart-Harris in 1939, the experiments being designed to show the transfer of the highly individual encephalitogenic character to other influenza virus strains.

The strain which I shall call neuro-WS has been maintained by Stuart-Harris (2) in mouse brain passage, and it is now highly adapted to that situation although it is still fully pathogenic both for the chick embryo and by the intranasal route for the mouse. If one compares neuro-WS with the strain developed by mouse lung passage, WSM, there are several well marked differences. I think it is worth while tabulating these to indicate: (a) what a wide range of "marker" character-

istics in the genetic sense is now available for influenza viruses, and (b) what considerable differences can arise between two substrains derived from the same original stock. The differences of particular value are the pathogenicity of neuro-WS for the mouse intracerebrally and for the chick embryo, the inability to produce the indicator form by any manipulation we have tried, the very weak enzymic activity of neuro-WS, and the extreme heat resistance of the haemagglutinin action of WSM.

Although Stuart-Harris developed the neuro-strain from one initially adapted to chorioallantoic membrane passage, as it exists after stabilization in the mouse brain, the allantoic cavity is essentially a new host tissue. It might be expected, therefore, that on adapting this mouse brain virus to the allantoic environment, changes in its character would occur. We therefore made 14 successive passages of neuro-WS by the allantoic route in each of two series. In one, passage was by 0.05 ml. of $1:10^{-8}$ dilution of the preceding fluid; in the other 0.05 of 1:100 dilution was used. The 10^{-8} dilution was virtually the limiting dilution; at nearly every passage a few negative embryos were obtained.

The results were strictly analogous to those with the O-D change. Passage at limit dilution gave fluids all showing the original characters of neuro-WS. Passage at 10^{-2} showed an irregularly progressive approach to more "normal" characters. The most definite changes were the development of capacity to take on the indicator state and concomitantly to show enzymic action by destroying the normal allantoic fluid inhibitor. There was some indication of increasing heat resistance of the haemagglutinin and when the final low dilution passage fluid was passed this time at limiting dilution, one of the resulting fluids resisted heating to 54°C.; the other showed the normal destruction. It should be noted however that despite these changes the re-isolated low dilution virus showing capacity to become indicator, enzymic activity and increased heat resistance of its haemagglutinin had retained its full encephalitogenic power. None of the unusual *in vitro* qualities of neuro-WS thus seem to be necessarily related to its neurotropic quality.

The importance of this experiment is simply to show the working of the same principle that passage in a new host at limiting infective dilution will allow maintenance of original qualities that would be lost by passage at low dilution.

RECOMBINATION OF CHARACTERS

In recent years very great interest has been aroused by various indications that there were latent possibilities of the combination or interchange of qualities of two different strains of organisms giving no evidence of any form of sexual differentiation. I will omit any consideration of the primitive types of sexual fusion in the fungi though I feel certain that this field will eventually have to be brought into relation with the phenomena I am going to discuss. What I am concerned with are the phenomena observed with bacteria and viruses. The field was opened up by the observation of Griffiths (3) that when heat killed S pneumococci, say of type 2, were inoculated into a mouse along with living R pneumococci derived from type 1, the mouse was liable to die from pneumococcal infection—the strain isolated being S type 2.

You are all aware of the brilliant work by Avery and his collaborators (4) in showing how the transforming factor was present in what was by all available criteria pure desoxyribonucleic acid. Put somewhat crudely the R form had lost a part of its genetic mechanism which it appeared, could be replaced from without by one particular pattern of nucleic acid present on some of the molecules of the purified desoxyribonucleic acid. Essentially similar findings have been reported by Boivin for *B. coli*. More recently Tatum and Lederberg (5) have used *E. coli* variants "marked" by their special requirements for some amino acid or vitamin but otherwise capable of synthesizing all their metabolic and structural components. If two strains each requiring 2 factors, say A-B- and C-D- respectively, are mixed and the resulting culture plated on minimal medium, i. e. with medium on which only the parent prototrophic strain can grow; any colonies that appear must be derived from a recombination.

This conclusion of course is valid only when it is shown that such reversion to the prototrophic form by either of the original variants alone either does not occur or occurs with a frequency far lower than in the mixed culture. By suitable experiments it could also be shown that other patterns of recombination than the prototroph could be obtained. Their conclusion was that either there is a process of (morphological) sexual fusion with segregation of genes in *E. coli* or, much less likely, transforming principles of the pneumococcal type are liberated into the culture medium and that these can induce change in both directions.

With bacterial viruses rather similar evidence of recombination can be obtained. In brief, double infection of single bacterial cells is produced. When multiplication is completed, the cells burst—liberating a new brood of virus. By suitable manipulation it is possible to arrange that all the phage particles from a single "burst" will produce discrete plaques on a plate spread with an appropriate bacterial culture. The phage present in each plaque can then be characterized by appropriate tests. By using for the double infection two strains with suitable genetic "markers," evidence for recombination can often be obtained by simple inspection of the plate. The types of marker used are the morphological character of the plaque, the necessity or otherwise of tryptophane and Ca ion to produce lysis and the range of action on heterologous bacterial strains. From the evidence of Luria, Delbrück and others (6) it is widely believed that a phage like T2 (or C16), when it enters the susceptible host cell, breaks down into perhaps 20 subunits (genes) and that these units undergo a series of replications at the expense of material and energy in the cell. Only toward the end of the cycle are these subunits again aggregated into the characteristic infective units. In this reconstruction it is necessary to assume that one example of each gene is built into each new infective unit. The marker differences between 2 generally similar types of phage presumably result from the existence of certain structural differences between one or more of the corresponding genes of the two types, equivalent to allelomorphs in higher types of genetic system. When therefore two such phages multiply in a single bacterial cell, the final reconstruction of the infective units is likely to show some redistribution of the variant genes. A phenomenon equivalent to linkage is ascribed by Hershey and Rotman (7) to varying degrees of diffusibility of different genes. From the evidence presented it is hard to see any alternative interpretation of the process of bacteriophage multiplication other than that summarized by Luria (8) and outlined above.

There are obvious difficulties in carrying out experimental work with any animal virus at all analogous to the procedures used in phage work. It is known that on occasion two different viruses can invade a single cell and both produce characteristic morphological expression of their multiplication therein. There are many more instances in which one virus may interfere with the action of another in a way which

would suggest that the presence of a virus of low pathogenicity in a cell will prevent the multiplication within that cell of the second more virulent virus. Two virus particles may enter a single cell, but I know of no practical technique by which all their progeny liberated on necrosis of the cell can be identified. In any practicable type of experiment on recombination of the characters of two animal virus strains, one must infect a tissue containing many cells with relatively large amounts of virus. In due course one will obtain from this tissue a population of virus particles the composition of which will be determined by the relative success of the different types (original types, mutants and recombinants) in multiplying intracellularly, in finding further opportunities to infect fresh cells and in surviving till the virus-containing tissue extract is finally tested by the experimenter. Further, it is only possible under ordinary circumstances to *isolate* from a mixture the dominant strain present, and this is of course almost always likely to be one of the original strains used. What is required then is to arrange circumstances so that *both* of the original strains used are prevented from multiplying while some possible recombinant is enabled to do so.

My first attempt to do this was made in 1942. It was unsuccessful but illustrates the type of set up required. Newcastle disease virus (NDV) and a normal strain of influenza A virus MELB, both grow well in the allantoic cavity; NDV invades the embryo and produces haemorrhagic lesions in the brain and elsewhere containing much virus. Attempts were made to protect the embryo with anti-NDV serum in the yolk sac with the thought that only a recombinant combining the invasiveness of NDV with the serological character of influenza would reach the embryo brain.

The method failed, and I doubt very much whether the one that we have found successful would ever have been evolved by simple logic. It arose from studies primarily directed toward the variation of virus inhibitor in brains infected with neuro-influenza virus. Since the neuro-WS strain has virtually no enzymic action *in vitro*, attempts were made to see whether the addition of an enzymically active non-neurotropic strain would increase the destruction of inhibitor in the infected brain. We found that in suitable proportions the addition of a normal influenza virus to the neuro-WS would render the mixture non-encephalitogenic. This is of course a typical example of interference and had

previously been reported as such by Vilches and Hirst (9). If a standard rather small dose of neuro-WS is mixed with serial dilutions of non-neurotropic mouse strain of WS and each mixture inoculated intracerebrally in mice, one finds first complete interference then a zone of partial interference and delayed death and finally the unmodified lethal action of neuro-WS.

In some mice at the zone of partial interference small amounts of virus only can be detected by subinoculation of the brain to eggs and some of these fluids have the character of a new strain combining two dominating characteristics, the neurotropism of neuro-WS and the extreme heat resistance of WSM haemagglutinin. In these mice multiplication of neuro-WS was inhibited by the interference produced by the nonneurotropic strain. WSM is intrinsically unable to multiply in nervous tissue, and the recombinant therefore had a chance to appear. From four different mice, virus of what we call WS N-M character was obtained and shown to be capable of repeated reisolation from limiting infective dilutions with retention of the double character.

Similar experiments with mixtures of neuro-WS and the standard strains of MEL and SW resulted in isolation of the corresponding recombinants neuro-MEL and neuro-SW. Here the task of isolation could be simplified by making the mouse brain dilutions in anti-NWS serum before inoculating chick embryos. On at least two occasions the recombinant was isolated from an excess of neuro-WS virus by this method.

It might well be objected that very great trouble would need to be taken to ensure that these alleged recombinants were actually pure clones and not merely mixtures of the original strains. In practice we have found that the recombinants have also shown characters which differ from those of either "parent." The WS N-M strain is much further to the right of the receptor gradient than either neuro-WS or WSM. Neuro-MEL and neuro-SW are both convertible to the indicator form by simple heating which is ineffective on MEL, SW and NWS. In all cases it has been shown that all essential characters remain demonstrable in allantoic fluids derived from limit infective dilutions both of allantoic fluid and mouse brain material. Whatever may be the interpretation there is nothing in the least equivocal about the experimental facts.

It is impossible, however, to avoid speculating on what the phenomenon does mean. Does it make it necessary to assume that influenza virus multiplication follows much the same pattern as has been postulated for the bacterial viruses? I should like to use the final portion of this lecture for a brief and necessarily superficial discussion of the process of influenza virus multiplication.

THE MULTIPLICATION OF INFLUENZA VIRUS WITHIN THE CELL

The experimental facts that we must take note of include: (A) the evidence from the Henles' work (10) of "burst" liberation of free virus from infected cells strongly suggesting that most virus is set free by necrosis of the infected cell; (B) the temporary disappearance of demonstrable virus when it has been taken up by living susceptible cells (Hoyle, 11, Isaacs, 12, Henle, 13) with the somewhat uncertain evidence for the appearance of soluble complement fixing antigen before the reappearance of infective virus; (C) the facts of interference between two living viruses or the blocking of infection by the use of killed virus; especially the evidence that interference is not a cell surface action but an intracellular one (14); (D) the existence of influenza A viruses in which the standard morphological form is a filament of extremely variable length (15); (E) evidence for an accumulation of ribonucleic acid in nucleolus and cytoplasm in the early stages of virus infection of the cell (16); (F) the recombination phenomena described above with due note of the fact that it becomes apparent at the point where interference is taking place.

Our present working hypothesis is frankly a simple adaptation of that adopted for bacterial viruses by Luria (6) in 1947 and first suggested in relation to influenza virus by Hoyle (11). We postulate that an influenza virus particle is adsorbed to the cell surface by the mechanism described in the first lecture and is then taken into the cell. There, as a result of some interaction with cell components, the virus particle disaggregates giving rise to a certain number of separate genetic units. It is impossible to guess how many such units are derived from each particle. An influenza virus particle is considerably larger than a T2 phage particle but contains much less desoxyribonucleic acid. Possibly the number of units is close to the 25 postulated by Luria for the bacteriophage. As in the case of the phage it is by no means clear how

much of the virus particle is made up of these genetic units and how much can be regarded as structure built up under genetic control but not itself of genetic character.

When the genetic units are free within the susceptible cell, they become centres of self-replication by appropriate association with some host-cell source of energy and building stones. Toward the end of the period of infection new formation of infective particles begins perhaps by the segregation from the pool of genetic units of groups comprising all those forming the complement of a single particle and their subsequent cooperation in the reconstruction of the infective organism. It is difficult to know how far it is justifiable to elaborate this picture of what is taking place within the infected cell. I think, however, that if a consideration of what may be happening provides a useful working hypothesis which allows the formulation of a practicable experimental approach, that is an adequate justification for going somewhat beyond the bounds of established fact. There is further the consideration that in many ways viruses may be regarded as uniquely effective "probes" of intracellular structure and function and that information and ideas derived from their use may be valuable to cytologists not directly familiar with the virus field.

There are some features of the particular system—influenza viruses in the mouse brain—with which we have worked which provide a further stimulus to look for a more comprehensive working hypothesis. I have concentrated on the evidence for the occurrence of recombination between two viruses, but I have also mentioned the readily demonstrable phenomena of interference. Using the same system Schlesinger (16) has recently described the effect of non-neurotropic viruses on the brain cells. He finds evidence that although such viruses do not produce infection in the ordinary sense, there is invasion of cells, destruction of virus inhibitors and a single cycle of virus "multiplication" which gives rise not to infective virus but to two incomplete products—haemagglutinin and complement fixing antigen.

To bring these phenomena into the picture we have to elaborate it in some such way as follows: It may be suggested that in the course of evolution each type of the postulated genetic units must have become adapted to make effective contact with some cellular entity capable of providing its material and energetic needs for replication. In the most

general terms this may be regarded as one of the "lock-key" relationships analogous to those concerned in antigen-antibody or enzyme-substrate interactions. To make the suggestion a little more concrete we may assume that the virus gives rise to four sorts of "genes," **A B C D**, with probably a number of allelic genes of each type. **A** genes require to make contact with the corresponding cellular component *a*, **B** with *b*, and so on. When this is established, there will be a flow of energy and material from *a* to allow the replication of the virus unit. This may be expected to activate the cell's synthetic mechanisms to produce more of *a* and so set in progress a concentration of cell activity toward the replacement and increase of those parts of its mechanism being used by the virus units for their replication. Eventually, of course, this must lead to exhaustion and disorganization.

Where a virus unit is capable of being adsorbed to the corresponding cell component, but either because it has been physically damaged (lethal mutation) or because of a slightly incorrect "fit" cannot effectively draw nutrient from it, we have a situation which might well account for the phenomena of interference. Suppose we have two viruses of basically similar genetic structure but in relation to *a* of a given type of cell their **A's** differ so that $\mathbf{A}_1$ makes ready effective contact while $\mathbf{A}_2$ makes contact but cannot utilize that contact. If the second virus enters a cell first its $\mathbf{A}_2$ units may unite with and render unavailable all *a* components. A subsequent entry of the first virus will thus find no *a* components available for its $\mathbf{A}_1$ units, and irrespective of what takes place in regard to **B C D** or of the fate of the cell, no viable infective virus can be liberated from the cell. The findings in the mouse brain can be fitted directly to this scheme if we assume that neurotropism depends on the fact that **A** in the neurotropic strains is properly adapted to the nerve cell *a* while the non-neurotropic viruses have a unit of the type $\mathbf{A}_2$. **B, C, D** can multiply, and from the pool of virus units incomplete forms can arise; but in the absence of **A** components they are noninfective either for the mouse nerve cell or for the chick embryo.

In other situations the conditions are probably less simple, and there well may be instances where the difference in effectiveness of $\mathbf{A}_1$ and $\mathbf{A}_2$ in making contact with the cell component *a* is quantitative rather than absolute. There is also plenty of evidence that many cells are in-

fected by virus which is nominally pathogenic for that particular cell type, yet do not undergo necrosis or reliberate virus. On the microscopic as on the macroscopic scale there must be the same inverse relationship between virulence of the parasite and host resistance. With a virulent virus the proportion of cells infected which undergo necrosis and liberate virus will be higher than with one of lower virulence and with influenza viruses these differences may be extreme. It may eventually be found that each individual "gene" has in a real sense its own "virulence," and that when the process of infection and necrosis is completed, in addition to infective virus particles there will be present a variety of noninfective virus material built up from those genes which were produced in excess of the slowest multiplying component. In addition to Schlesinger's results in the mouse brain, one should also refer to the finding of von Magnus (17) that under certain conditions large amounts of haemagglutinating but noninfective virus may be liberated into the allantoic cavity. The complement fixing antigen which is interpreted by Hoyle (11) as the noninfective multiplying form of influenza might well represent one or more types of genetic unit produced in unnecessary abundance.

The process of reintegration of infective units which must be postulated at the close of the phase of replication of the subunits, is very difficult to picture in physico-chemical terms, but the difficulties are no greater than confront any other attempt to visualize organized living structure in such terms. Those of you with a direct interest in virus research will have seen the beautiful pictures by the Hampstead workers of filamentous forms of influenza and fowl plague viruses. Here we have at least a prima facie refutation of any statement that virus multiplication must be by binary fission. It seems to me that one can fit the idea of a breakdown into subunits with resynthesis of the infective form very readily to the production of a continuous nonparticulate form in which there is no necessary definition of any infective individuals.

I have presented our evidence for the occurrence of recombination in the particular situation of the neurotropic influenza viruses. In any genetic sense this is observational rather than experimental material. If we adopt the hypothesis I have outlined, in what experimental directions should it be possible to build up further evidence of its truth or otherwise? If there is, in fact, a particulate basis of virus in-

heritance, is it in any way possible to interpret phenotypic appearances (i.e. the properties of a clone in the form of allantoic fluid) in terms of a genotypic pattern or will we be forced to conclude at this level that phenotype and genotype are the same?

My visit overseas has forced us to postpone any attempt to go more deeply into these matters, but as I see it at the moment, the only practicable line of approach is the accumulation and characterization of as many recombinant strains as possible from some suitable pair of parent strains. It is our intention on returning to the field to take neuro-WS and our stock MELB viruses as the most promising material and to extend considerably the experiments on the conditions determining interference between the two strains. When the conditions which most readily result in the appearance of recombinants have been ascertained, the logical thing will be to follow in as much detail as possible the multiplication and/or disappearance of the parent strains and the quantitative features of the appearance of recombinants. There is already evidence that an unstable neuro-MEL strain of low encephalitogenic action may on one mouse brain passage take on the character of what we are coming to regard as standard neuro-MEL. It is more than likely that all primary recombinants are subject to intensive selection for survival and that what is isolated from mice at the 5th or 6th day is essentially the result of successive mutation and perhaps secondary recombination in a cell infected by both parent strains. With proper manipulation including the use of monovalent immune serum it might become possible to isolate in pure allantoic fluid culture some of the early unstable recombinants.

I have so far discussed recombination in influenza viruses as no more than an interesting intellectual problem of the same status as recombination in bacterial viruses. But it is hard to avoid wondering whether we are not coming near to something with very real practical potentialities for human weal or woe. We have shown that it is possible to transfer to one influenza virus defined by its serological character the specific *virulence* of another, the capacity to infect fatally a tissue on which it had previously no action. This seems to offer in a more real sense than previously the opportunity for finding what actually determines the virulence of a virus and by implication how that virulence may be overcome.

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LECTURE III

THE ECOLOGICAL APPROACH TO THE COMMON VIRUS DISEASES OF TODAY

It is almost obligatory in speaking in general terms of infectious disease to point to the successes which in this century have resulted from our dealings with most of the important diseases of the past. The great plagues—bubonic, yellow fever, cholera, smallpox and typhus appear no longer in civilized countries. Malaria, typhoid and dysentery are steadily being eliminated, and the septic infections are under therapeutic control. A combination of two sorts of scientific work has brought about this result. On the one hand there has been the combination of the objective study of clinical disease, the development of bacteriology and virology in the laboratories and the study in the field of how the pathogens survive in nature and how they are conveyed to their human hosts. On the other hand we have the less logical but even more effective approach by which a combination of accident, empiricism, the resources of modern chemistry and the American capacity for organized investigation, has provided a whole series of agents with specifically lethal effects on this or that group of organisms which in one way or another restrict the fulfillment of human desires.

In the third lecture however I want to look not at our successes but at our failures, at those diseases which flourish as much in the middle of the twentieth century as at any previous period. One of them and the one with which I shall be most concerned is influenza; a second is the group of respiratory infections including and typified by the common cold; the third is poliomyelitis. I am interested to know whether there are any common features of these three diseases which can account for our failure to influence their incidence either by the general improvement of living standards or by more specific public health measures.

It is always worthwhile, I think, to approach any disease from the fundamental ecological point of view of asking how does the parasite concerned survive in nature? Twisting that question slightly to fit our present topic we may ask, what are the characteristics of a pathogenic microorganism which will best fit it for successful survival as a parasite of human beings living in modern urban communities?

The requirements are:

(1) That small numbers of organisms can readily establish infection in some accessible region of the body.

(2) That descendant organisms are freely liberated into the environment in such a way that they can pass readily to other hosts. This involves two factors: (A) liberation in the secretions of mouth or nose or very free liberation in faeces and (B) the continuance of the host's normal activities and movement among his fellows.

(3) That following infection immunity is not sufficiently solid to prevent subsequent recolonization by the organism.

These requirements are all fulfilled in one way or another by the three diseases I have mentioned, and a more detailed discussion of the problems they present can be built up on this framework of the requirements for optimal survival.

1. Ease with which infection is acquired on contact with the microorganism

All the infections we are dealing with are due to viruses. The influenza viruses can now claim to be the best known and most adequately studied of all the viruses infecting man. The poliomyelitis viruses are also well known and readily identifiable although technical difficulties prevent the same elaboration of experimental attack that can be applied to influenza. In regard to the virus or viruses responsible for colds and other minor respiratory infections, we still await the discovery of an adequate experimental technique. We can say on the basis of human experimentation that they are due primarily to small filterable microorganisms of the same general quality as other viruses but very little more.

Epidemiological evidence indicates that where previously uninfected populations are concerned all three diseases are very readily passed from person to person. Persons who have been isolated for six months

or more in Arctic regions will regularly contract a respiratory infection soon after their first contact with civilization. There are many records of outbreaks of influenza or poliomyelitis arising in "virgin soil" communities and rapidly involving a high proportion of the population.

This raises the question of the mode by which these viruses initiate infection in susceptible subjects. Broadly speaking there are two ways in which virus infection can be initiated. Any obligate intracellular parasite must find means of entering susceptible cells. If a virus has a relatively wide range of cells within the host that it can parasitize, the commonest mode of entry is by minor trauma which can place it in such a situation that it will be taken up passively by the wide variety of cells with potentially phagocytic powers. Examples of traumatic virus infection include herpes simplex, vaccination, yellow fever and the mosquito borne encephalitides, lymphogranuloma venereum, and in a somewhat different sense psittacosis where the virus particles are probably taken up by alveolar cells. Where viruses can infect untraumatized mucous membranes, it appears necessary to look for more active mechanisms by which normal superficial cells can be invaded. In the first lecture I described the phenomena associated with haemagglutination by influenza virus. From the biological point of view I think we can regard this mechanism for the specific adsorption of influenza virus to the cell surface, as an adaptation to allow infection of an intact cell surface. The function of the enzymatic mechanism is not so clear. It seems certain that virus can be taken into the cell without the necessity of its subsequent release from the receptor, but I should still be inclined to think that it plays a part in allowing the initiation of those phases of the infective process that follow entry into the cell.

We can say nothing about the virus of the common cold, but there are distinct hints that the poliomyelitis viruses may have somewhat similar relations to cells. It is certain that one of the polio-like viruses of the mouse GDVII agglutinates human red cells (1), and preliminary reports of its inhibition by a polysaccharide extracted from intestine have appeared. Work on haemagglutination by other neurotropic viruses is in active progress and I shall be surprised if in a few years time workers with such viruses have not obtained evidence of an important part being played by the interaction of virus and cell surface.

At the risk of making a premature generalization it is, I think, a

good working hypothesis to look for entrance mechanisms in those viruses which can readily infect intact mucous membranes or more generally produce infection by "simple contact."

The liberation of infective material into the environment

In a modern community we can eliminate arthropod-borne infection and the grosser forms of faecal contamination. In the case of a virus there is no possibility of multiplication in milk or any other non-living part of the environment. The obvious mode of transfer is by dispersal into the air directly or indirectly of mouth and nasal secretions containing the virus. This may take the form of droplets liberated by sneezing or talking or of dried droplet nuclei. Contaminated dust may be secondarily dispersed from floors and ledges or from textiles contaminated in various ways, especially bed clothes and handkerchiefs. By inhalation infective particles can lodge anywhere in the throat and reach either the lower respiratory passages or the alimentary tract. In respect of poliomyelitis where the evidence of easy contact infection appears to be inescapable there seem to be two possibilities for consideration: the first, droplet spread as for respiratory infections; the second, which does not seem to have been considered as closely as perhaps it should be, the transfer of faecal infection by way of fingers and mouth. The two outstanding features of the epidemiology of poliomyelitis, the very extensive and relatively prolonged liberation of virus in the bowel and the necessity for close association between children in the transfer of infection, seem to be brought together better on this assumption than on any other.

The second requirement for effective dissemination of infection is that the shedder of virus should continue to "get around." If a patient is hospitalized from almost the moment he becomes infective for others, the virus in question is not going to infect more than an unlucky nurse or doctor before it is eliminated from further chance of survival. In a recent smallpox experience in Britain a rather large number of hospital personnel were infected by an undiagnosed case of smallpox. When the situation was realized and each nurse or attendant isolated at the onset of symptoms which is generally held to be one to two days before the development of significant infectivity for others, no further cases occurred (2).

Similarly we hear of small outbreaks of pneumonic plague and of infections by psittacosis group viruses in which the victims are limited to one patient infected from an animal source and a sequence of sick bed attendants of one sort or another. Such highly lethal epidemics never spread freely simply because the virus shedder is too sick to move about amongst his fellows.

In other words effective spread is only possible when a significantly high proportion of infections are mild or subclinical but associated with free shedding of virus. In the case of epidemic influenza it is well known that subclinical infections occur, as evidenced most frequently by the observation of an antibody rise but also on a number of occasions by the demonstration of virus in throat washings from the contacts of persons with clinical influenza (3). There may well be another type of distribution of influenza virus. The most likely explanation of the old conundrum of where influenza goes in the summer is that there can occur trickling streams of subclinical infection in the virtually complete absence of clinical infection and certainly of any overt epidemic. Incidents pointing in such a direction are the episodes where influenza has broken out in some isolated community following the arrival of apparently healthy individuals from outside. We have recently described something of this type from Ocean Island in the central Pacific Isaacs et al. (4). Here in October, 1948, the arrival of a ship carrying a contingent of Chinese laborers was followed a little over a week later by the outbreak of a severe epidemic of influenza A involving the Micronesian population who formed the other moiety of the labor force and some of the small European group but none of the Chinese. Similar occurrences have been quite frequent on Ocean Island, in fact some form of respiratory infection follows the arrival of each new contingent of Asiatics, which normally happens once a year. In October, 1949, there was a smaller outbreak, and this time we could find no evidence of influenza viruses being involved.

There are some very interesting problems involved in the subclinical case of influenza. Why does a person who liberates virus freely into his throat and who develops as marked an antibody rise as most clinical cases, show in these instances no symptoms whatsoever? In 1940 I infected a group of 17 volunteers with influenza virus (5); three of these had virtually no antibody against the infecting strain, and

these were the only ones who showed unequivocal evidence of infection. Two of them had typical acute attacks of clinical influenza; the third had no symptoms and normal temperatures throughout the period but subsequently showed a very sharp antibody rise. The difference then does not seem to be associated with the presence or absence of antibody at the time of infection. Liberation of virus in quantity must be associated with cell necrosis and breakdown and it is usual to regard symptoms as manifestations of absorption of toxic products of cell disintegration. I can see no immediate answer to the problem, but it seems possible that a closer study of the processes of virus multiplication and lesion production in the mouse lung may be helpful. In the early stages of adaptation one can often obtain almost as much virus from lungs which are infected without consolidation as in the later stages from lungs which are or will eventually be totally consolidated.

Even more difficult and interesting problems arise in regard to the relation between clinical, i.e., paralytic and subclinical cases of poliomyelitis. In this case the question is not so highly relevant to the problem of liberation of virus. I think everyone is in agreement that the presence or absence of paralytic infection is immaterial to the liberation of infective virus in the throat secretions or in the intestinal contents. What we urgently require to know is the nature of the cells in which the infection that leads to liberation of the virus takes place. As far as I am aware there is no experimental evidence whatever to let us know where the virus found in the faeces is generated. One would guess cells of the intestinal epithelium, but if this is true, it must be an infection which only in very rare instances results in cell necrosis since there are no macroscopic lesions of the bowel mucosa. The other possibility is that some relatively rare type of cell accessible from and to the bowel lumen is involved—one thinks perhaps of autonomic nerve cells immediately beneath the mucosa. One would imagine that this problem could be studied effectively with one of the mouse viruses of the Theiler group that appear to behave ecologically very similarly to human poliomyelitis virus.

I have recently been in some contact with an important practical problem of poliomyelitis which arose last year in Australia. This was the appearance of paralytic poliomyelitis involving especially a limb

innoculated a short time before with pertussis or combined pertussis-diphtheria vaccine (6). This is in no way immediately relevant to my subject, but it has stimulated me to think a great deal about the factors which may convert a subclinical infection into a paralytic one. That is, of course, the most important problem that is presented by any disease whose standard expression is clinically unrecognisable but in which the unusual form is a severe or fatal illness. In addition to poliomyelitis, meningococcal infection may be taken as another example. The most direct approach to an understanding may, I think, be made from a consideration of the *age incidence* of the clinical disease.

One of the most interesting recent descriptions of a poliomyelitis epidemic was of that which occurred last year amongst Eskimos in Canada (7). Probably all members of the community were infected, there was a paralysis rate of 40 per cent and a death roll of 15 per cent —but no infant under 3 was involved, and there were a number in the community. It is fair to assume that this group of individuals had had no past experience of a virus of this immunological type—that it could be called a "virgin soil" community. Now in other presumptive virgin populations poliomyelitis has also produced paralysis much more extensively in adolescents and young adults than in infants. The only epidemic of this type that I know of which has been carefully reported is one occurring in St. Helena in 1947 (8). Here the brunt of the attack was fairly evenly distributed over the ages from 5–20, with much less in children under 5 and diminishing amounts in the older age groups. In heavily endemic communities it is well known that there is a great concentration of paralytic cases in infants while older children escape. Here immunological phenomena undoubtedly play a part. Confining ourselves to previously unexposed communities and extrapolating perhaps a little freely from the evidence we can, I think, express a useful working hypothesis by a diagram like Figure 1. The family of curves shown is intended to express the proportion of persons infected at each age who will be diagnosed as suffering from paralytic infection. The curves from above downward are for strains of progressively diminishing virulence, i.e., capacity to invade the central nervous system.

This is essentially an hypothesis, and I have perhaps introduced it into a lecture on influenza virus infections without any real justifica-

tion. However, I do think that in attempting to clarify one's ideas on the influence of immunity on the epidemic behaviour of any infectious disease, it is highly desirable to express the *disease*-producing power of the infection at different ages. The mortality according to age may

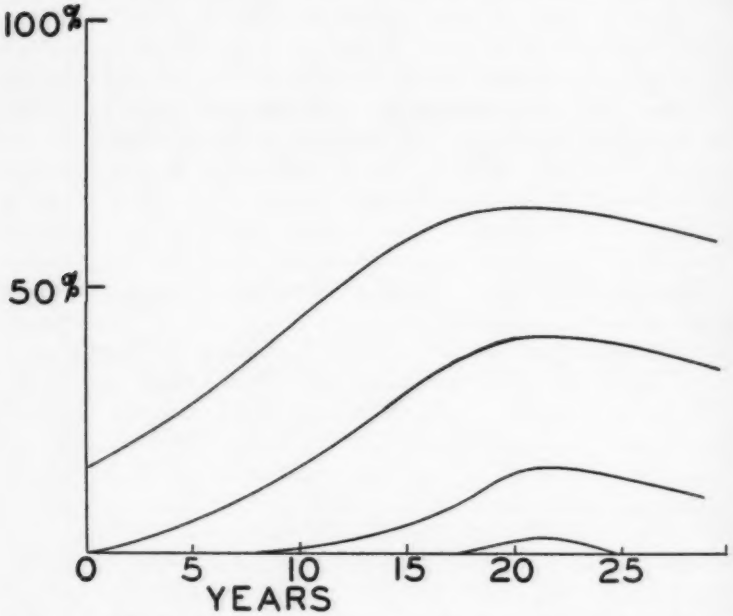

FIG. 1. A family of curves to represent the incidence of paralysis in human populations of normal age distribution. All individuals are presumed to be infected with poliomyelitis virus and to have had no previous contact with the disease. The lines indicate the results to be expected with strains of diminishing invasiveness from above down. Ordinates: percentage paralyzed; Abscissas: years of age.

be the only convenient index of severe disease, and if this were used instead of paralysis, the peak of each curve would be lower and shifted toward a higher age group. It must be stressed that for a given disease there will almost always be required a family of curves and not a single curve. Except for such apparently stable infections as mumps and measles, different epidemic strains of most viruses and pathogenic

bacteria may vary widely in their virulence using this difficult term as characterising the average clinical intensity of the disease they produce, in non-immune individuals.

The shape of these curve families clearly varies greatly from one type of infection to another, but it is only rarely that actual events provide information of any directness. From the very nature of the situation the impact of any well known type of disease on a wholly unimmunized population is both rare and difficult to observe with accuracy. With rarer diseases an occasional opportunity for accurate observation arises, but the age incidence observed must always be closely scrutinized to determine whether age may not have influenced the likelihood of infection as opposed to the intensity of effect produced after infection.

The type of curve showing the greatest influence of infection in the young adult age group as seen in poliomyelitis is of particular interest in view of the experience of the 1918–19 influenza pandemic. I think one of the most striking epidemiological phenomena on record is the sudden change in the age incidence of death ascribed to influenza that took place in England in the second week of June, 1918. Throughout the period of the pandemic and with only slight variations in pattern in different parts of the world the major incidence of death was on the young adult from 20–35 years. There has never been a wholly satisfactory explanation of this phenomenon. There is nothing to support the suggestion that those who had been through the 1889–92 epidemics of 28 years previously were immune and most young adults non-immune. Nor does it seem likely from the epidemiological character of the pandemic that it could be regarded as due to a wholly new antigenic type of virus. The actual incidence of clinical infection was no higher (20–30 per cent) than may be found in many benign influenza epidemics—the difference was only in the lethality of such an undue proportion of the infections. Even in 1918–19 subclinical infections were much commoner than clinical, and we can feel reasonably certain that the dissemination of infection was largely the responsibility of those who were not forced to their beds.

This discussion has taken us rather far from our starting point: that a flourishing common infection must have an easy means by which the infective agent can be passed to the largest possible number of

potential susceptibles. It is an evolutionary necessity for both species, host and parasite, that the subclinical or near subclinical infection should be the norm of the interaction between the two and that this should result in relatively free liberation of fresh broods of parasite.

Virus workers particularly are liable to be rather obsessed in their experimental work with obtaining highly virulent virus strains and new highly susceptible hosts. For all sorts of purposes these are highly desirable, but when taken along with accounts of classical and therefore unusual epidemics, they tend to induce a wrong emphasis on the nature of infectious disease. I have tried here to stress the wide range of problems presented by this fact of subclinical infection which to me dominates the whole of epidemiology insofar as it is concerned with *stabilized* host-parasite relations.

The role of immunity in common virus infections

The third of our requirements for success in a twentieth century virus is that following first infection immunity should not be so substantial as to preclude any subsequent recolonization by the organism. Measles is in many ways a very successful virus—few of us reach adult life without being attacked—but once that attack is over, it is probably completely exceptional for either clinical or subclinical infection to occur again.

Of the three virus diseases I have taken, the common cold is the one that best exemplifies this point. All experimenters have found that their volunteers are again normally susceptible to deliberate infection three months after a cold. Common experience, however, suggests that there are at least very great variations in the ease with which clinical infection results from exposure and that these are immunologically determined. Andrewes (9) stresses that while instillation of relatively large amounts of infective material into the nose will produce colds in about 55 per cent of his English volunteers, it is extraordinarily difficult to produce colds at will by any form of normal association between infectors and infectees. Everything suggests that all or most dwellers in normal communities maintain a substantial basic immunity against colds, by the fact of repeated subclinical infection with the virus or viruses concerned. There is a continual traffic in such viruses that only ceases in small groups who

are completely isolated from outside contact for months or years. On the reestablishment of outside associations such isolated individuals are extremely susceptible to clinical colds. It is probably useless to discuss the mechanism of this short lasting immunity in the absence of any effective laboratory approach, but the phenomena should be borne in mind when immunity to influenza is being considered.

There is no lack of observational and experimental data about immunity in influenza, but there are still plenty of problems. It may be helpful to think of influenza as lying mid-way between the infections like measles and mumps which give long-lasting specific immunity and the common cold with its transitory resistance needing constant restimulation.

In thinking about any problem involving immunity to virus infection it is worthwhile keeping well in front of one, two generalizations which are probably true and certainly useful. They are: 1) that all immunity against viruses is mediated by antibody and 2) that antibody is effective only when a sufficient concentration can come into contact with virus at a point before it infects those cells, damage to which is responsible for the characteristic symptoms of the disease. Immunity against measles on this view is so extremely effective because on the one hand the first infection results in a viraemia which ensures a potent stimulus to the whole antibody-producing mechanisms of the body and on the other that if a second infection with the virus occurs, symptom production cannot occur except by the passage of free virus through the blood stream where it will be exposed to a maximal concentration of antibody.

In influenza the mechanism of immunity is probably of a very different character. Infection is of the respiratory epithelium and spreads from cell to cell over the surface, i.e. by diffusion in the thin film of fluid lying on the free surface of the epithelium. The significant concentration of antibody is that which is present in this liquid film at the time infection is spreading from the point of initiation to adjacent cells.

Some direct evidence of this has recently been obtained by Fazekas (10) in Melbourne in his study of the conditions in experimental influenza of the mouse. He found that immunity as measured by the direct challenge of graded infection was dependent on the presence of cir-

culating antibody but was more directly related to the titre of antibody obtainable in bronchial washings than to that in the serum. If mice were immunized with an intranasal injection of vaccine, they were more solidly immune than after an intraperitoneal injection that produced a similar antibody rise. Suitable analysis showed that this was not due to local antibody production but to an effect of the intranasal injection in allowing a greater mobilization of antibody into the surface film of liquid. If for instance mice are immunized intraperitoneally with an influenza A vaccine, and this is followed by an influenza B vaccine intranasally, there is a greatly increased resistance to challenge with influenza A. But if the intraperitoneal vaccination with the A vaccine is omitted and only the B vaccine given intranasally, there is no immunity at all against experimental A infection. The vaccine given intranasally acts only by increasing the amount of antibody that can pass through on to the respiratory surface.

If we hold to our first generalization that virus immunity is wholly a matter of antibody we can now, I think, see rather more clearly what are the factors which determine natural immunity in influenza.

If we start with an individual, a child who has never previously been infected with influenza A, we can presume the first infection results in a mild clinical infection. There is passage of virus and virus products to bronchial lymph nodes and perhaps to more distant centres of antibody production. Antibody of at least two types makes its appearance in the serum: 1) complement fixing antibody corresponding to a soluble product of cells infected with influenza virus, and having no known function in regard to active immunity and 2) antibody or a series of antibodies which are related to the surface of the infective virus particle and which appear to be responsible both for *in vitro* neutralization tests and for active immunity in the animal.

In the second lecture I said something of the significance of the small complement fixing antigen and its corresponding antibody—the reaction is both of great theoretical interest and of first rate practical value in epidemiological work—but it probably has no relevance to the problems of immunity in the infected person or animal. Here we are concerned only with the antibodies corresponding to the surface components of the infective virus particles themselves.

When such an individual is again exposed to influenza by inhalation

of a few particles of infective virus, we can assume that say a dozen bronchiolar epithelial cells are infected. The virus multiplies and from some of these cells a brood of new virus particles is liberated into the superficial film. Infection (in the sense of demonstrable clinical or subclinical disease) will result if the process of spreading superficial cellular invasion and necrosis can continue. This will be determined by whether or not there is sufficient antibody in the film to prevent or slow down successive infection by the particular type of virus which is encountered. It is essential that the antibody should be of the right type.

In spring, 1945, we had the interesting experience of watching a mixed influenza A and B epidemic in a school (11). There were three instances in which boys had two distinct clinical attacks at an interval of two to three weeks. Serologically all were A and B (or B and A) and from one boy we isolated both viruses. There were no double A or double B attacks. This is rather striking evidence of the complete specificity of immunity as between A and B influenza viruses.

There is a less well marked but still important difference in effectiveness of immunity between the subtypes of influenza A. The most striking example of this was the complete failure of a vaccine containing previously current strains of influenza A virus to protect against influenza A in February 1947 although a similar vaccine had been effective against the influenza of 1943–44. In the latter epidemic Salk *et al.* (12) had shown that if a large group of individuals were studied for the antibody content of their serum, there was a good correlation between antibody level against strain *Weiss* and resistance to clinical influenza but a much poorer correlation with antibody against the standard strain PR8. Weiss was a strain of the serological subtype characteristic of the epidemic.

In Melbourne we have been watching influenza since 1935, and like everybody else in the field we have found serological differences amongst the influenza A strains that we have isolated. And like everyone else we are still puzzled as to how to handle, classify and interpret such differences. Since we must primarily be concerned with the human problem, I should be strongly predisposed to use human immune sera obtained after clinical infection as the important type of reagent for the serological differentiation of strains. In practice,

however, we come up against some major difficulties. It is impossible in any given infection to know what former attacks of influenza the subject had experienced and the antibody response may well have been influenced by such past experience.

Very early in the history of serological studies on influenza viruses it was observed that adults might show antibody to swine influenza virus as well as to typical influenza A strains, but that in children under 12 this swine-type antibody was not found. Later it became clear that the "breadth" of (antibody) response to a given infection varied widely between individuals, children giving as a rule more specific antisera than adults, specific being used in the sense that the homologous strain is much more readily inactivated than any heterologous strain. Large numbers of reports have been published on the homologous and heterologous activity of convalescent human sera, and I have no doubt that most influenza laboratories have like our own a great deal of unpublished data of the same type. In general the results appear variable and confusing but very frequently one obtains the impression that the current influenza A virus of any epidemic is different from the strains that were responsible for former epidemics in the sense that antisera from the older strains do not effectively neutralize it. On the other hand, the new strain will often produce in human beings a strong antibody rise against the older strains. Largely as a result of discussion with Anderson on unpublished work of this type, I think that a working hypothesis can be evolved which will provide a practical basis for the organization of serological studies on influenza A viruses and for the modification where necessary of the strains to be used in vaccine preparation.

Our hypothesis is that immunity following an influenzal infection is quite high and long lasting against strains of the homologous type and against strains of types that have been present in the past. If influenza A virus strains can undergo mutation to serological types which are not readily susceptible to the characteristic antibody of the community, they will clearly have a survival advantage over the strain responsible for the preceding epidemic. It would be reasonable to expect therefore that from one epidemic period to another there should be a change in the serological character of the virus. Oversimplifying the situation, it appears that influenza A virus survives by

constantly building serological novelty on to its past antigenic structure.

We can picture as it were a successive modification of surface which in some way covers over but preserves what was past antigenic surface. The result of contact with antibody will be determined only by the surface structure; the antigenic effect as stimulus to antibody formation will vary from one host to another, but in those capable of a broad response buried antigenic qualities may be manifested. It is in accord with the known history of influenza A to believe with Shope (13) that SW (1931) may represent the serological type of the 1918–19 pandemic, that throughout the single epidemiological unit of the populous areas of the world there has since been a series of mutations sorted out for survival from the influenza A stem in which serological character has been constantly changing. At certain points in the sequence the difference became striking enough to demand the acceptance of a new subtype. Using the practical criterion that a ferret immune serum shows less than 10 per cent reaction against a heterologous strain, there are, as I have mentioned, SW, WS, MEL-PR8 and A^1 subtypes, but there have been many variants and intermediates.

The practical human criterion of difference is whether a vaccine made from strain X will protect against an epidemic due to strain Y. Translated into specific procedural terms we should incorporate into our current influenza vaccine, any new strain which is inadequately neutralized by average sera from subjects experimentally immunized with the current vaccine. When a major failure of influenza vaccines to protect occurred in February, 1947, it was subsequently shown that a sharp change in character had taken place from the former serological subtypes of influenza A (14). It is reasonable to believe that if the new strain had been in the vaccine, the same satisfactory result would have been obtained as in the previous A epidemic of 1943–44. To the obvious objection of the impossibility of knowing beforehand what the next influenza epidemic will be, there is the interesting answer that in this particular instance a group of A strains of the new subtype had been isolated in the previous year in Australia. The fact that winter is the common season for influenza and that all recent experience indicates that for influenza the whole civilized world is a single epidemiological unit, makes the relationship of the Southern and Northern hemispheres

very important. If as we are suggesting, influenza A strains are constantly changing in serological character, any outbreak in the Southern hemisphere will probably provide strains which are more like those which will be responsible for the next Northern epidemic than any stored strains from past epidemics in the North. In the same way Northern strains may be the most suitable for vaccination against the next Southern epidemic.

Is it possible on the basis of 17 years work on influenza viruses to offer an interpretation of the 1918–19 influenza pandemic which as Greenwood (15) pointed out was one of the three great plagues of history, killing a greater absolute number than any epidemic that preceded it and being exceeded in mortality rate only by the plague of Justinian's reign and the Black Death? Obviously any interpretation must be highly speculative in the absence of any real knowledge of the primary infecting agent. Nevertheless the immense importance of knowing whether we may expect another similar pandemic and if so how we should deal with it justifies and demands an analysis of what evidence is available.

So I shall offer a few suggestions. In the first place I believe that the primary agent was an influenza virus of type A and that there is a considerable likelihood that the Swine type virus isolated by Shope and Lewis in 1931 may be a descendant modified by passage in swine, of the responsible virus. The onset of the pandemic in April, 1918, was undoubtedly associated with a sharp change in the genetic character of the virus. New type influenza first appeared in an area—Western France—where very large numbers of American troops were mixing for the first time with European service and civilian populations, and there had been considerable influenza of normal type in both continents during the preceding winter. If the experiments on recombination that I described in the second lecture are significant, it seems to me that we must consider quite seriously the likelihood that in this mingling of strains from two parts of the world an opportunity for recombination occurred which provided a form of higher virulence than could have arisen by simple mutation. The history of the pandemic indicates that further changes of virulence and of serological character occurred during its course. When an influenza epidemic involves hundreds of millions of people, we have the production of perhaps 10^{20} individual virus particles—a population within which an enormous range of

mutation might be expected. To one interested in microbial genetics it is almost tragic to think that modern techniques were not then available to isolate and characterize the strains involved at different phases of the epidemic.

The characteristic change in the age incidence of mortality would at first sight suggest that the new virus was serologically wholly distinct from any former strain. There is little doubt that it did differ serologically to some extent from 1917 strains, but I think an additional quality of virulence must be postulated. If the virus could infect and necrose a somewhat wider range of cells perhaps the cells of the alveolar walls as well as the bronchiolar epithelium, then I think a reasonable explanation of the changing age-incidence of death could be provided.

This brings us back to the problem, exemplified equally in poliomyelitis, of what determines the particular shape of age-incidence curves of morbidity or mortality. In the most general terms they represent changing physiological backgrounds against which secondary results of the infective process are played out. Infection of any sort evokes a series of responses, local inflammatory changes mediated chiefly by pharmacologically active products of damaged cells, general changes in which the pituitary-adrenal system of hormones is largely concerned and the production of antibody by mesenchymal cell systems that have taken up the infective agent or its antigenic fragments. For optimal recovery all these processes must be of appropriate intensity, not necessarily of maximal intensity. The peak of mortality so often seen in young adult life seems in general to be related to the over-activity of local responses in infections involving widespread tissue damage. Physiological reactivity is at its height in young adult life, and the repair of local trauma and infection is then at its most efficient phase. The best recovery from generalized infections appears however in the child from 6–10 years of age.

During the pandemic on this interpretation, virus damage to the lungs plus secondary infection by current respiratory bacteria provoked unusually severe vascular and exudative reactions in the lungs. The intensity of these varied characteristically with age being sufficiently severe in the young adult to provoke death in a much higher proportion than was seen in younger or older individuals.

One final aspect of the pandemic of very great interest to the ecol-

ogist was its diminishing intensity with time in its attack on previously untouched populations. As far as I can determine the last sizable area of the civilized world to be infected was Tasmania which was first reached in August, 1919 (16). The mortality there was considerably lower than in any other Australian state or for that matter in any other country from which statistics are available. Some islands were missed for even longer periods, and when New Caledonia was infected in 1921, the influenza was of "normal" virulence. It seems to be a rule that has no exceptions that an exceptionally virulent epidemic is an unstable situation which quite rapidly returns to a tolerable equilibrium between host and parasite.

In influenza then we have a complex immunological situation. There are two quite distinct immunological types A and B behaving as quite unrelated diseases only the first of which has been fully studied. Immunity following attack is solid and probably long lasting against the same subtype, but it is overcome usually at 2–4 year intervals by the appearance of new serological variants. Subclinical infection is probably always at least as common as clinical.

Finally, a little may be said about the significance of immunity in poliomyelitis. Perhaps the most striking way of visualizing the effect of past subclinical infection is to compare the age incidence of paralytic infection in the two island epidemics of Malta, 1943 (17) and St. Helena, 1947 (8). In Malta the disease was practically confined to children under 5; in St. Helena about the same proportion of children under 5 were paralysed as in Malta; but far greater numbers of cases occurred in each of the three age groups: 6–10, 11–15 and 16–20, as well as considerable numbers in adults. St. Helena had probably not been infected by poliomyelitis at least during the last 20 years; Malta had certainly been an endemic centre for many years. There seems to be no other way of accounting for the absence of poliomyelitis amongst older Maltese children than by ascribing to subclinical infection in infancy a high capacity to induce subsequent resistance to paralytic infection.

The active immunological work on poliomyelitis of the last few years (18, 19) and particularly the findings with experimental infection in the chimpanzee seem to be providing a picture of a situation not wholly dissimilar to that of influenza. There are three immunological types of

poliomyelitis of which "Brunhilde" is of much greater clinical importance than the other two. Unfortunately the second type, "Lansing," is the only one that can conveniently be used for antibody titrations so that there are some awkward gaps in the data. It appears that nonparalytic infection is like influenza—essentially a surface infection, that it gives rise to antibody production and that the presence of antibody presumably on the intestinal mucosal surface prevents a subsequent superficial infection for some time at least. It also greatly diminishes, probably for a longer period, the likelihood that a paralytic infection of the CNS will follow implantation of the virus in throat or bowel. Immunity is probably type specific, but there are findings, Brown et al. 1950, (20) which suggest strongly that there are "broad" antisera capable of neutralizing heterologous as well as homologous type virus and that these are common in adolescents and adults.

In a community like Malta or any other city of relatively backward hygiene babies are commonly infected with virus of one or more types and mostly suffer a nonparalytic infection with free excretion of the virus in the faeces for a week or two. Antibody production will occur and provide a temporary protection against bowel infection with the same type and a more lasting immunity against paralysis. In such an environment repeated contact with the virus will give occasional mild intestinal reinfections and a more and more substantial immunity. When as in 1943 a more actively invasive strain appears of the same serological type as formerly, it will infect only those children who have not yet had the experience of less invasive strains needed to produce effective immunity, i.e. the infants of the community. With the highly invasive strain many more of these will show paralytic infections even if as is likely the actual transfer of virus from child to child is no freer than it was in former years.

Recent epidemics in North America and Australia or New Zealand have shown a different type of age incidence, much less in those under five than in Malta, a maximal incidence in children of 5–10 years and considerable numbers of adolescents and young adults. The difference is most simply interpreted on the basis that improving hygiene of infancy is postponing the average time of first contact with poliomyelitis till the period of early school life. At this age first infection is more liable to be paralytic than if it occurred in infancy. There will

however be a much larger number of nonparalytic infections the ratio depending on the intrinsic invasiveness of the current virus strain.

If this is a true interpretation, an extremely difficult problem faces the public health administrator. The improvement in standard of living and of hygiene which has reduced the incidence and severity of most other infectious diseases appears to be almost directly responsible for the increasing prevalence of poliomyelitis in the advanced countries of the world.

It seems to me to be one of those situations in which one has to make a deliberate choice between attempting to eradicate the disease or accepting the necessity of living with it. The first is obviously desirable if it can be managed, but if the attempt to do so introduces greater dangers than to retain the status quo, we should think very carefully before we act. If on the other hand we decide that it is necessary to accept the continuing existence and spread of the virus, then our only practical measure is to devise some means of ensuring natural or artificial immunization.

Any attempt to eliminate the spread of poliomyelitis by improvement of intestinal hygiene seems an impossible counsel of perfection. If in every polio season there is enough virus in general city sewage to be detected that should give us some indication of the extent of the problem and of how readily polio virus must be transferred from person to person. One suggestion that has been made is that some intestinal antiseptic should be devised which is not absorbed but which can destroy the virus in its extracellular condition so rendering infection both of the subject and of his contacts unlikely. This is by no means outside of the bounds of possibility and such a substance might be successful in cutting short an epidemic if it were properly used.

But the whole history of poliomyelitis as a disease emerging *pari passu* with the development of pride in decent living amongst the common people, of its changing age incidence, of its effect in very isolated communities impresses me with the terrible danger of having a community in which most young adults have never suffered from subclinical infections with the virus.

This lecture is concerned with three of the most successful of human parasites, parasites which for centuries have lived in a fairly tolerable association with the human species. If we are going to modify those

associations in any drastic fashion, we must beware of the consequences. The tolerability of the infections is in each case based on the possibility of sublinical immunization maintaining a continuing resistance against serious infection and the only legitimate action is to imitate and improve on the natural processes.

The logical approach from the ecological angle toward the prevention of poliomyelitis is either to find acceptable means of insuring a reasonable transfer of intestinal infections between children in infancy or to devise some means of effective immunization. The technical difficulties of obtaining a dead virus vaccine seem at the present to be insurmountable, and I feel confident that sooner or later it will become necessary to use living vaccines given by mouth in infancy, perhaps under cover of gamma globulin passive protection. But I think that someone will need more courage than I possess to initiate such a programme. So far I have concentrated on the immunological approach as the important means of preventing lethal or damaging effects of the common viruses. But if you look at the families of curves dealing with the age incidence of severe infections you will realize that there is a physiological as well as immunological approach.

In 1918–19 the same type of virus infecting a man of 25 and a boy of 10 had 10–20 times a greater likelihood of killing the man. In the Hudson Bay poliomyelitis epidemic the two year old baby was unaffected while the adult had a 40 per cent likelihood of paralysis. I do not think it is beyond the possibilities of modern endocrinology to determine why the young adult in 1918–19 (as it were) killed himself by too vigorous response to the same infection that the child took in its stride. With understanding it is usually possible in this field to provide a therapeutic interference that will tide over a short lasting emergency. I hope that some of those who understand the complexities of ACTH action will look critically at the clinical reports of death from 1918–19 influenza with its "heliotrope cyanosis" and decide what type of experimental and therapeutic study would be most likely to provide the knowledge needed if such an emergency arose again. Perhaps such work is already being done—in any case I can imagine no problem of more importance to medicine.

A similar approach to the problem of paralytic poliomyelitis has already begun. Why is the CNS of the baby apparently less vulnerable

than that of the non immune adolescent? A very simple answer may be merely that the muscular activity of the baby is less than that of the boy and that a vulnerable fatigued state of the motor cells of the cord does not often develop. Probably the full answer is more subtle than that but again I feel that there may be possibilities in the future of providing for the short period of emergency a pharmacological means of diminishing the vulnerability of nerve cells exposed to the threat of poliomyelytic infection. The studies of nerve cell metabolism in relation to poliomyelitis that have been going on in Baltimore (21) provide an important basis for the possible development of such methods. I can make no suggestions as to what form they may take, but as I have already hinted, our Australian experience of the onset of paralytic polio after immunization seems to offer a challenge. Here is one way in which the vulnerability of nerve cells can be increased —does that knowledge contain within itself the seed of how vulnerability can be decreased?

Perhaps I might conclude by expressing the hope that in these three lectures I have been able to indicate the lines along which virus research at the academic rather than at the practical level is progressing. Research on infectious disease has always had many aspects, but broadly they can be divided into the study of the parasite as such and of its interaction with the host. When virus disease is the topic, a special interest arises out of the very simplicity of the parasite; and at the present time there is a strong tendency to feel that many of the basic problems of biology can be best approached at the virus level.

I should again like to stress that in the first two lectures I was concerned with the interaction of the influenza virus particle with the individual host cell. This necessarily involves some consideration of the intrinsic nature of the virus, but the general approach is better expressed as an attempt to use the behaviour of the virus as a clue to cell structure and function. The work arising out of haemagglutination can be fairly claimed to have opened up a new approach to the function of mucopolysaccharides in the cell surface and to have provided a valuable biological reagent in the vibrio receptor destroying enzyme. The phenomena associated with virus multiplication within the susceptible cell are more complex and less clearly understood, but when taken along with the results of bacteriophage research seem to

offer one of the most direct approaches to the understanding of cytoplasmic function. Further correlated study of interference, recombination and "incomplete virus" production can hardly fail to provide a rich reward.

Finally in this last lecture I have tried to present an approach at the wholly different ecological level to influenza as an epidemic disease. I may or may not have been wise to include extensive references to the two other common virus diseases, poliomyelitis and the common cold. I hope at least that the discussion will indicate the value of the concept of survival as applied to the pathogen. In regard to the major human problem of the control of the harmful effects of diseases with which we are compelled to live, I have tried to show that there are two approaches—to imitate the natural process of immunization by subclinical infection and to find what are the physiological conditions which determine whether a given infection produces significant damage or remains at the subclinical level. Such knowledge may well become applicable to practical therapeutics.

Even with such outstanding successful pathogens as those with which I have been concerned, there is no reason for pessimism about our eventual ability to minimize their potentialities for harm.

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CERTAIN ASPECTS OF THE PHARMACOLOGY OF 3-HYDROXYCINCHONINIC ACID[1]

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Certain cinchoninic acid derivatives have been shown in previous communications from this laboratory (1, 2, 3, 4) to exhibit at least three pharmacological effects, namely, an antidiuretic action, a blocking of the renal tubular secretion of phenol red, and a stimulation of the anterior pituitary causing a decrease of the ascorbic acid content of the adrenal glands. It appears that in this series of compounds, antidiuretic activity is determined by the basic structure exemplified in 3-hydroxycinchoninic acid (2). This hitherto undescribed substance has now been synthesized and examined in regard to its antidiuretic activity, its ability to block phenol red secretion and its ability to stimulate the anterior pituitary.

PREPARATION OF 3-HYDROXYCINCHONINIC ACID

This substance was obtained by a three-step synthesis involving the preparation of 3-nitrocinchoninic acid, reduction of this substance to the corresponding amino acid and conversion of the latter to the desired 3-hydroxycinchoninic acid by thermal decomposition of its diazonium chloride. The structure of the final product was established by its ready conversion to the previously known 3-quinolinol.

The preparation of 3-nitrocinchoninic acid by condensation of isatic acid with β-nitroacetaldehyde oxime has been described in a German patent (5) and by Colonna (6). In our hands this author's modification of the patented synthesis was found to afford the desired product in relatively low yield. Accordingly, several pilot experiments were conducted with a view to bettering this yield. As a result of these experiments the procedure described below for the preparation of 3-nitrocinchoninic acid was adopted. Colonna (6) reduced this substance to 3-aminocinchoninic acid by means of iron powder and hot acetic acid. This same reduction is more conveniently effected with ammonium sulfide and the crude product of the reaction is more easily purified. Preliminary experiments upon the conversion of 3-aminocinchoninic acid to the desired 3-hydroxycinchoninic acid disclosed that both diazotization of the former and the subsequent thermal decomposition of its diazonium salt proceed more readily in dilute hydrochloric acid

[1] This investigation has been aided by a grant from the U. S. Public Health Service, and by a grant from the Life Insurance Medical Research Fund.

than in dilute sulfuric acid. This is probably a consequence of the relatively low solubility of the sulfate of both 3-aminocinchoninic acid and of its diazonium derivative.

3-Nitrocinchoninic Acid. The following reaction was conducted in a 1 l. 3-necked flask equipped with an electrically driven stirrer, dropping funnel and thermometer and arranged so that an external ice-water bath could be easily brought into position to control the temperature of the contents of the flask during the course of the reaction. A solution of 234 gm. of 85.8 per cent potassium hydroxide in 340 cc. of water was introduced into the flask and the stirrer started. During the course of approximately 30 minutes, 61 gm. (1 mole) of nitromethane were added through the dropping funnel at a rate such that the temperature of the reaction mixture was easily maintained within the limits 25° to 30° by appropriate manipulation of the ice-water bath. After the addition of the nitromethane, and after the spontaneous rise in temperature had ceased, the reaction mixture was heated to 40° and held at this temperature for 2 minutes. Then, while the temperature was held between 40° and 45° a second portion of 61 gm. (1 mole) of nitromethane was added dropwise to the mixture. Following this addition, the mixture was heated to a temperature of 50° to 55°, held within these limits for 8 minutes and cooled to room temperature. The solution of β-nitroacetaldehyde oxime so obtained was poured slowly and with continuous stirring into a cold solution prepared by adding a solution of 210 gm. of 85.8 per cent potassium hydroxide in 200 cc. of water to a suspension of 308.6 gm. (2.1 mole) of isatin in 200 cc. of water contained in a 3 l. flask. The resulting mixture was left at room temperature for 25 hours, then poured slowly into a continuously stirred mixture of 700 cc. of hydrochloric acid (sp. g. 1.19) and 8100 cc. of water. The resulting mixture was stirred for an hour and then left at room temperature for 48 hours. The solids which separated during this time were collected in a 24 cm. Buchner funnel and washed with four 100 cc. portions of water. The combined filtrate and washings were concentrated in vacuo (see below). The moist filter cake was broken up and stirred continuously for 1 hour with a solution of 85 gm. of sodium bicarbonate in 1200 cc. of water. This mixture was filtered and the undissolved solid (isatin β-oxime) was washed with five 50 cc. portions of water. The combined filtrate and washings were transferred to a 4 l. beaker and treated dropwise with 200 cc. of 6 N hydrochloric acid while continuously being stirred. The tan colored solid thus precipitated was collected in a 75 mm. Buchner funnel, washed with water and allowed to dry, first in air, then *in vacuo* over calcium chloride. It weighed 46.1 gm.

The filtrate and washings of this product were added to the original filtrate undergoing concentration and the volume was reduced to approximately 1500 cc. This concentrate after standing at room temperature over night deposited a further quantity of solids, which were separated, washed with a little water and then stirred for 1 hour with a solution of 30 gms. of sodium bicarbonate in 500 cc. of water. The insoluble fraction was separated and the filtrate therefrom was treated dropwise with 60 cc. of 6 N hydrochloric acid. After a few hours at room tem-

perature the precipitated product was collected in a 60 mm. Buchner funnel, washed with three 5 cc. portions of water, and dried to constant weight (21.8 gm) in vacuo.

The two lots of crude 3-nitrocinchoninic acid obtained in this manner were combined (67.9 gm.) and dissolved by gentle warming in a solution of 34 gm. of 85.8 per cent potassium hydroxide in 500 cc. of water. Ethanol (675 cc.) was added to this solution, which was left in an ice-box over night. The potassium salt of 3-nitrocinchoninic acid which separated from the mixture, when collected, washed with four 5 cc. portions of ethanol, then with two 5 cc. portions of ether and dried at 105°, weighed 48.3 gm. The mother liquors from this salt were freed of ethanol by distillation, made acid to Congo red by the addition of hydrochloric acid and left over night at room temperature. The dark brown pasty solid which separated was collected, dried on a porous tile, triturated with two 10 cc. portions of ethanol, transferred to a Buchner funnel, washed with two 5 cc. portions of ethanol and dried in vacuo. This material, from which a great deal of the color had been removed by treatment with ethanol, weighed 18.3 gm. It was dissolved in a solution of 8.8 gm. of potassium hydroxide in 140 cc. of water, 175 cc. of ethanol were added, the mixture left in an ice-box over night, then filtered. The solid product, when washed with four 5 cc. portions of ethanol and dried at 105°, weighed 9.8 gm. This was combined with the original crop of the potassium salt and the total amount (58.1 gm.) was dissolved in 625 cc. of hot water. The resulting solution was stirred with 2 gm. of decolorizing carbon for 5 minutes and filtered. The hot filtrate was treated with 50 cc. of 6 N hydrochloric acid and left over night at room temperature. The precipitated acid, collected on a Buchner funnel, washed with four 5 cc. portions of water, and dried at 105°, weighed 47.4 gm. (Yield = 21.7 per cent on the basis of the weight of nitromethane used in the synthesis). This pale yellow crystalline product melted with decomposition at 200–201° and proved to be satisfactory for the preparation for 3-aminocinchoninic acid. Upon recrystallization from boiling ethanol (31 cc. per gm.), the product decomposed at 203–204°, in agreement with values previously recorded for the decomposition point of 3-nitrocinchoninic acid (5, 6).

3-Aminocinchoninic Acid. In a 500 cc. flask equipped with a stopper carrying a thermometer, Bunsen valve and gas inlet tube connected to a tank of hydrogen sulfide, 60 cc. of ammonia water (sp. g. 0.9) were carefully added to a suspension of 32.7 gm. (0.15 mole) of 3-nitrocinchoninic acid in 80 cc. of water. The flask was placed in an ice bath. When the temperature of its contents had fallen to 5°, hydrogen sulfide was admitted through the gas inlet tube and passed into the mixture until all of the 3-nitrocinchoninic acid had dissolved and the solution was saturated with the gas. The contents of the flask were then transferred to a 600 cc. beaker, diluted with 40 cc. of water and boiled until free of hydrogen sulfide and excess ammonia. The hot reaction mixture was filtered, and the warm filtrate was continuously stirred while 40 cc. of 6 N acetic acid were added. The mixture was cooled to room temperature and filtered. The product was washed with two 5 cc. portions of water, suspended in 250 cc. of warm water, brought into solution

by the dropwise addition of a slight excess of 6 N ammonia, and filtered. The filtrate was stirred, treated with 12 cc. of glacial acetic acid, and left over night. The bright yellow product was collected in a Buchner funnel, washed with four 5 cc. portions of water and dried in vacuo at room temperature. The 3-aminocinchoninic acid (25.4 gm. 90 per cent of the theoretical) melted with decomposition at 225–226° and was used without further purification for the preparation of 3-hydroxycinchoninic acid. A portion recrystallized from glacial acetic acid melted with decomposition at 229–230°.

Analysis Calc. for $C_{10}H_8O_2N_2$: N = 14.87% Found: N = 14.82%

3-Hydroxycinchoninic Acid. A solution of 28.2 gm. (0.15 mole) of 3-aminocinchoninic acid in a mixture of 600 cc. of water and 40 cc. of hydrochloric acid (sp. g. 1.19) was cooled to 0° by means of an ice-salt bath and diazotized in conventional fashion by the dropwise addition of a solution of 10.4 gm. of sodium nitrite in 50 cc. of water while the temperature of the reaction mixture was held below 12°. The solution of the diazonium salt thus obtained was heated to 35–40°, held within this range of temperature for 1 hour, then heated to 70° and held at this temperature until the evolution of nitrogen had ceased. (During this process higher temperatures must be avoided since they result in a materially lower yield of the desired end product, probably as a result of decarboxylation.) As the thermal decomposition of the diazonium salt proceeded, crude 3-hydroxycinchoninic acid separated as very small reddish brown crystals. The reaction mixture was cooled and filtered; the product was washed first with water, then with alcohol, dried first at 37°, and then in vacuo at room temperature. This crude product weighed 22.7 gm. (80 per cent of the theoretical). It was suspended in 700 cc. of water and brought into solution by the addition of the theoretical quantity of 6 N sodium hydroxide. The resulting dark colored solution was stirred for ½ hour with 2 gm. of decolorizing carbon and filtered. The filtrate was heated to 70° and, while continuously stirred, made acid to Congo red by the addition of 6 N hydrochloric acid. A further 10 cc. of 6 N hydrochloric acid was added and the mixture was left over night. The difficultly filterable non-crystalline product so obtained was separated from the mother liquors by centrifugation. The sedimented product was washed successively with four 10 cc. portions of water and two 20 cc. portions of acetone and dried, first in air at 37°, then in vacuo over sulfuric acid. Yield, 22.1 gm. of a deep yellow powder. When the melting point of this substance was determined in the conventional manner, it was found to decompose at 212° to 213°; when the sample was inserted in a bath preheated to 205° and the temperature was increased at the rate of 2° per minute, decomposition occurred at 213–214°.

As 3-hydroxycinchoninic acid is essentially insoluble in the common organic solvents it was recrystallized from warm 5 N hydrochloric acid (5 cc. per gm.) from which, upon cooling, it slowly separated as readily filterable minute yellow prisms. Two such recrystallizations did not change the decomposition point of the substance.

Analysis. Calc. for $C_{10}H_7O_3N$: N = 7.41%. Found: N = 7.33%.

3-Quinolinol. A mixture of 1.9 gm. of 3-hydroxycinchoninic acid and 5 cc. of nitrobenzene was refluxed for 2 hours. The nitrobenzene was removed by steam distillation and the remaining aqueous suspension was evaporated to dryness. The solid so obtained was heated on the steam bath with 25 cc. of 3 N sulfuric acid. The resulting solution was treated with decolorizing carbon and filtered. Upon cooling a mass of orange crystals separated from the filtrate. These were separated, dissolved in a minimum volume of hot water and 10 cc. of 1 M potassium carbonate was added to the solution. The mixture was cooled to room temperature and the pale yellow solid which separated was collected, washed with water, dried briefly on a porous tile and recrystallized from hot 60 per cent ethanol from which it separated as fine colorless needles which darkened upon exposure to air for a short time. After drying at 105° for 10 minutes this substance melted at 199.5 to 200° and this melting point was not depressed by admixture of the substance with an authentic sample of 3-quinolinol prepared from technical 3-aminoquinoline by the method of Mills and Watson (7). These investigators state that 3-quinolinol melts at 198° while others (8) cite this value as 196° to 198°.

Analysis. Calc. for C_9H_7ON: N = 9.65%. Found: N = 9.70%.

PHARMACOLOGICAL EFFECTS

The methods used for testing the activity of this compound as an antidiuretic agent, as a blocking agent for phenol red secretion, and as a stimulant of the anterior pituitary have been described in previous publications (1, 3, 4).

Acute Toxicity. None of nine mice injected subcutaneously with 800 mgm. per kgm. of this compound as the sodium salt died, while nine out of twelve mice died from an injection of 1600 mgm. per kgm. Since 400 mgm. per kgm. of 3-hydroxy-2-phenylcinchoninic acid (HPC) is fatal to about one-half of the mice injected (2), it is apparent that the introduction of the phenyl group markedly increases toxicity.

Antidiuretic Activity. In a dog in which a water diuresis had been produced, injection of 5 mgm. of either HPC or 3-hydroxycinchoninic acid per kgm. caused no antidiuretic effect, while in the case of both compounds, the injection of 10 mgm. per kgm. produced an unequivocal effect. A second dog responded to 10 mgm. but not to 5 mgm. of 3-hydroxycinchoninic acid per kgm. and to 5 mgm. but not to 2.5 mgm. of HPC per kgm. The new compound is about equal in antidiuretic activity to HPC.

Effect of Phenol Red Secretion. The rate of excretion of phenol red was measured with and without previous intravenous injection of 3-hydroxycinchoninic acid or HPC. Results are given in table I. It

is apparent that 3-hydroxycinchoninic acid is about as active as HPC in blocking the renal tubular secretion of phenol red.

TABLE I

The Effect of 3-Hydroxycinchoninic Acid on Urinary Excretion of Phenol Red

DOG NO.	HOURS	MILLIGRAMS OF PHENOL RED IN URINE				
		Control	HPC		3-Hydroxycinchoninic Acid	
			10 mgm/kgm	20 mgm/kgm	10 mgm/kgm	20 mgm/kgm
14	½	2.25	1.57	0.86	1.51	
	1	3.17	2.69	1.56	2.68	
	2	3.98	3.58	2.45	3.89	
32	½	2.77		0.74		0.60
	1	3.84		1.70		1.35
	2	4.55		2.90		2.13

TABLE II

The Concentration of Ascorbic Acid in the Adrenal Glands of Rats Two Hours after Subcutaneous Injection of Derivatives of Cinchoninic Acid

Name of Compound	Dose	No. of Rats	Concentration of Ascorbic Acid
Normal Rats			
	mgm/kgm		*mgm/100 gm. Adrenal*
Controls	—	13	408 ± 16.8*
3-Hydroxy-2-phenylcinchoninic Acid	200	4	217 ± 33.8
	100	10	307 ± 21.6
	50	4	442 ± 8.8
3-Hydroxycinchoninic Acid	200	4	247 ± 53.0
	100	10	330 ± 14.8
	50	6	364 ± 19.1
Hypophysectomized Rats			
Controls	—	8	329 ± 14.2
ACTH	10	4	199 ± 11.2
3-Hydroxycinchoninic Acid	200	6	321 ± 22.3

* Each figure given in this column is the mean with its standard error.

Stimulation of the Anterior Pituitary. Male rats of the Sprague-Dawley strain, weighing 200–280 grams, were used. The hypophysectomized rats of the same strain weighing 170–190 grams were used twenty-four hours after operation.

The results are summarized in table II. One group of hypophysectomized rats was given adrenocorticotropic hormone (ACTH) to demonstrate that adrenal ascorbic acid could be reduced from the control levels. The data indicate that 3-hydroxycinchoninic acid caused a marked decrement in the ascorbic acid content of the adrenals of normal rats, but not of hypophysectomized rats. Its activity as a stimulant of the anterior pituitary is of the same order of magnitude as that of HPC.

SUMMARY

A synthesis is described for the preparation of 3-hydroxycinchoninic acid. This compound is less toxic acutely than 3-hydroxy-2-phenylcinchoninic acid, but has approximately the same activity as an antidiuretic agent, as a blocking agent for phenol red secretion, and as a stimulant of the anterior pituitary gland.

We wish to thank Frances Fallon, Irene T. Payne and Sara Ann Verplanck for technical assistance.

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MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

Hurd Memorial Hall, The Johns Hopkins Hospital
Baltimore, Maryland

Monday, November 13, 1950, at 8:15 p.m.

Dr. Fisher: Ladies and gentlemen, this being our first meeting since our great victory at the polls last week, I thought it would be appropriate if our Society were to go on record, first of all, in expressing our appreication to all of you who have done so much to help put across the fight that we were fighting; secondly, to thank the Sunpapers for the part they took in the campaign from start to finish in every issue; and, finally, I thought we should pay special tribute to Dr. Chesney and thank him for the work he has done in organizing and bringing this whole thing through to a successful conclusion.

Thank you.

The first paper on the program is by Drs. T. B. Turner and D. H. Hollander and will be presented by Dr. Turner.

CORTISONE IN EXPERIMENTAL SYPHILIS*

"Under the influence of cortisone therapy, syphilomas induced in rabbits by intradermal or intratesticular inoculation of Treponema pallidum show striking alterations from the picture usually observed in experimental syphilis, in that the lesions become soft and spongy, there is an excessive accumulation of a mucoid material tentatively identified as hyaluronic acid, treponemes are unusually abundant, and the production of Wassermann reagin seems to be inhibited. Upon withdrawal of cortisone there is a rebound phenomenon in which the lesions reach an unusual size."

Dr. Fisher: The papers this evening all deal with the same general subject, but do handle different aspects of the problem, and I think it will be best to ask for discussion of each one as we deal with it. Is there any discussion of Dr. Turner's paper?

Dr. Chesney: Is the observation of non-healing of wounds under the influence of cortisone a new one?

Dr. Turner: No, that has been observed by others. The inability of the animal to form fibrous tissue in a normal manner is apparently due to altered behavoir of the ground substance, in which hyaluronic acid, instead of being converted to the

* This paper was published in the November, 1950 issue of the Bulletin of the Johns Hopkins Hospital.

related compound, chondroitin sulfuric acid, accumulates in excessive amounts. We know that hyaluronic acid is present in small amounts in normal skin and in certain other tissues, such as the walls of blood vessels. It is commonly present in large amounts in umbilical cord and in many sarcomas.

Dr. Chesney: Is there an increase in the incidence of generalized lesions in cortisone-treated animals?

Dr. Turner: We have not observed an unusually high incidence.

Dr. Fisher: Is there any other discussion?

I would like to ask Dr. Turner one thing. In his rabbits with experimental syphilis treated with cortisone, the lesions are all more or less well localized. I wonder what part the type of host plays in that? For instance, in experimental tuberculosis the results are quite different; at least, in preliminary experiments that have been reported in guinea pigs treated with ACTH the lesions have been more widespread, and the local lesions more diffuse and spreading than in the controls.

Dr. Turner: I cannot answer that question: The indurated tissues of a typical syphiloma, characterized by the presence in excessive amounts of this mucopolysaccharide which seems to be mostly chondroitin, may play an important role in localizing infection, whereas excessive amounts of the more mucoid hyaluronic acid may have the opposite effect.

Dr. Fisher: Is there any other discussion?

The second paper is by Drs. A. R. Rich, I. L. Bennett, T. H. Cochran, P. C. Griffith and D. C. McGoon; to be presented by Dr. Rich.

THE EFFECT OF ACTH AND CORTISONE UPON EXPERIMENTAL ANAPHYLACTIC GLOMERULONEPHRITIS

Lantern slides were presented demonstrating that acute diffuse glomerulonephritis, that reproduces with fidelity the pathological characteristics of human acute glomerulonephritis (marked proliferation of the endothelium and epithelium of the glomerular tufts, hemorrhage from the tuft capillaries, crescent formation, adhesions between the tuft and Bowman's capsule and eventual partial or complete organization of the more severely damaged glomeruli), can be produced experimentally as a result of the presence of foreign protein in the circulation of anaphylactically sensitized rabbits. The degree of nephritis varies, in different animals, from simple endothelial proliferation to a marked grade of damage exhibiting all of the above-mentioned alterations.

Treatment with ACTH greatly reduced the incidence of nephritis and maintained the glomeruli of the great majority of the animals in their normal state.

Treatment with cortisone suppressed all glomerular alterations of the type characteristic of ordinary acute glomerulonephritis, but the majority of the treated animals developed severe, focal glomerular lesions of a quite different type, characterized by the formation of "aneurysms" of the tuft capillaries often with rupture and hemorrhage, or with hyalinization resulting in the formation of large, often globoid hyaline masses in the tufts which, in some instances, were indistinguishable from the hyaline, globoid masses familiar in the Kimmelstiel-Wilson glomerular lesions in diabetics. There is some evidence that treatment with cortisone, alone, in the absence of sensitization, can damage glomerular capillaries. The failure of ACTH to produce glomerular lesions of the type produced by cortisone may be due to differences in dosage. The cortisone-treated animals developed marked lipemia, in which both the total fatty acids and cholesterol participated. No such marked lipemia resulted from the ACTH treatment. The deposition of glycogen and fat in the liver was also more marked in the cortisone-treated animals.

Dr. Fisher: Dr. Rich's paper is open for discussion.

Dr. Harvey: I would just like to ask Dr. Rich if he knows what the incidence of glomerulonephritis is in human reactions to horse serum.

Dr. Rich: Of course, we have had very little chance to study that. I think I should emphasize in most of the rabbits the glomerulonephritis is slight.

Dr. Mirick: I think both the papers—Dr. Turner's about the effects of these drugs on infection and Dr. Rich's on hypersensitivity states—might lead one to wonder what effects, if any, ACTH or cortisone treatment might have on the production of antibody by an animal, since antibody might play an important role in both of these conditions.

We have recently completed some studies in human beings to find out what effects ACTH and cortisone might have on the production of antibody. We have vaccinated fifty-nine individuals with pneumococcal polysaccharides. About half of them were treated with one or the other of these two drugs, and half of them served as controls. When antibody titers were measured there seemed to be no difference between the two drugs, but the patients who received either of them developed a significantly higher titer of antibody than did the controls.

As Dr. Turner pointed out, the experimental studies in animals in this respect have been quite a mixup as to what effects the drugs might have. I think this mixup can be unraveled a little bit if one thinks about the reported findings by species.

We found a drop of globulin in the sick patients during treatment with ACTH and, to a somewhat lesser degree, with cortisone, and this finding confirms that of others, who have studied human beings. Li and Rinehart, in their study of rats, reported similar findings. When treated with ACTH there was a rise in albumin and a fall in globulin. There have been similar changes in antibody production in rats to those we have found in humans. Adrenal stimulus in rats brings an increased production of antibody and adrenalectomy a decrease. In rabbits, how-

ever, almost all reported evidence shows the opposite to be true. Adrenalectomized rabbits produce more antibody than normal rabbits and those receiving ACTH or cortisone produce less. The degree of this reaction varies with different authors and perhaps that is due to the difference in the size of the dosage of the drug used or the duration of treatment. At any rate, I would like to make the point that there is a clear-cut species difference and that the rat and human being appear to be more alike in this respect than the rabbit and human being.

I will not spend any time here on hypersensitivity, as there is not enough known. Apparently, rabbits and humans react in similar fashion with regard to dermal sensitivity. In our patients using pneumococcal polysaccharides immediate dermal sensitivity was suppressed under ACTH or cortisone treatment and similar results have been found in rabbits. With the anaphylactic hypersensitivity of guinea pigs this is not at all true. It is reported that neither active nor passive anaphylaxis can be blocked by ACTH or cortisone. The point of my remarks is that what is found in one animal species need not apply to another. Obviously, there are many other points in the chain of events, besides the formation of antibody or the reaction of antibody with antigen, which may explain the results which both Dr. Rich and Dr. Turner have reported.

Dr. Futcher: Dr. Fisher, I would like to ask Dr. Rich if he has tried any agent other than ACTH and cortisone in preventing the experimental nephritis. A young Swiss investigator, named Reubi, in 1946 produced experimental nephritis by the Masugi technique and found that an antihistaminic agent called *Antistin* made by Ciba appeared to decrease the severity of the nephritis and I wonder whether Dr. Rich has had any experience with drugs of that type?

Dr. Rich: We haven't tried them at all, Dr. Futcher.

Dr. Brown: I am sure all of us have been equally impressed by the brilliant clarity with which Doctor Rich has demonstrated the complete pathological parallelism between the experimentally produced hypersensitivity type of nephritis and human glomerulo-nephritis. The effect of ACTH and cortisone in preventing the development of the experimental nephritis lends further support to the growing evidence that one of the main aspects of cortisone activity is its effect on the immune mechanism.

We have been carrying out clinical studies on the hypersensitivity mechanism which we believe is basic in the closely related rheumatic disease problem. Although our approach has been different, our thesis is in general accord with that of Doctor Rich. Cortisone promotes an early rise in albumin as well as a fall in globulin determined electrophoretically. We have found that intravenous injections of human serum albumin in rheumatic diseases may also promote a rise in albumin and a fall in globulin. Kunkel has noted that human serum albumin depresses the turbidimetrically determined gamma globulin levels in serum from patients with

liver disease and we have observed the same effect with sera from patients with rheumatic disease. It appears to us that serum albumin may not only play an important role in the hypersensitivity mechanism, but it may also provide an additional tool to probe the basic process.

For a number of years we have been investigating the possible role of pleuropneumonia-like organisms (L organisms) as antigens in the basic mechanism of rheumatic and other collagen-vascular diseases. During the past two years we have compared the clinical and ***in vitro*** effect of the newer anti-L substances, aureomycin, chloramphenicol, and terramycin with the original anti-L substance, gold. All of these substances may produce a selective intensification or flare-up of the rheumatic or collagen-vascular process in the areas of existing clinical activity suggesting an additional local release of antigen. It is of particular interest that terramycin, which has the most marked anti-L effect ***in vitro***, produces the most intense flare-up. Cortisone will suppress or completely block this induced flare-up phenomenon. Also, a complete cortisone block of a flare-up regularly induced with aureomycin, may be only partial with much smaller doses of terramycin. It appears to us therefore that the extent of the hypersensitivity reaction is probably related more closely to the existence and level of tissue antibody than to circulating antibody.

We therefore suggest that the high gamma globulin which may be observed in various rheumatic and collagen-vascular diseases probably reflects in a large part at least disturbed protein formation rather than the development of specific antibody.

I wonder if Doctor Rich would care to comment on the question of tissue antibody as compared to circulating antibody in his studies.

Dr. Turner: I would like to ask Dr. Rich whether he has made stains for metachromatic material in the hyaline accumulations in the kidney glomeruli shown so beautifully in his sections. Is it possible that this material may be mucopolysaccharide in nature, with transformation of one compound to another under the influence of cortisone?

There are accumulating indications that many of the so-called fibrous tissue reactions are the result of disordered metabolism of the mucopolysaccharides composing the ground substance. In response to some stimulus, bacterial or other, firm chondroitin-like tissue is built up; under the influence of cortisone and related hormones, this excessive mucopolysaccharide material is converted to hyaluronic acid which is soft and mucoid in character. I am wondering whether changes of this sort might be going on in the glomerular lesions under the influence of cortisone.

Dr. Fisher: Is there any other discussion?

Dr. Rich: I cannot answer Dr. Brown's question in its entirety. I am sure that he is better able, from his own excellent studies, to supply an answer, as far as that is possible in the present imperfect state of our information. I think that

it is clear that the amount of circulating antibody is not an accurate measure of whether visceral lesions will develop when antigen enters the circulation. The reactivity of different individuals to the same antigen may be very different, although all may possess the same amount of antibody. Some may develop lesions in one "shock tissue" and some in another; and others, equally sensitized, may develop no lesions. In tuberculin-type sensitivity, in which sensitizing antibody is not demonstrable in the plasma, there is very strong evidence that sensitizing antibody is attached to cells.

In reply to Dr. Turner, we have not made any studies of the hyaluronic acid content of the glomerular lesions in the cortisone-treated animals. I can say that neither the manner of formation of the hyaline masses nor their appearance suggests that they represent accumulations of hyaluronic acid. We expect to study these lesions in more detail histochemically.

Dr. Fisher: The last paper is by Drs. R. A. Lewis, R. Klein, L. I. Gardner, J. F. Crigler, Jr. and C. Migeon. It will be presented by Dr. Wilkins.

THE TREATMENT OF CONGENITAL ADRENAL HYPERPLASIA WITH CORTISONE

The effects of cortisone in the treatment of seven patients with congenital adrenal hyperplasia are reported. Five of these patients had evidences of excessive androgen secretion without any electrolyte disturbance, two showed excessive androgen secretion and a marked electrolyte disorder of the Addisonian type.

The administration of cortisone invariably caused marked decrease in the excretion of urinary 17-ketosteroids. Bioassays made in one case showed that the output of biologically active androgen was decreased to an even greater degree than the 17-ketosteroids. Urinary "estroids," determined chemically, and estrogens measured by bioassay, were high before treatment and were also reduced by cortisone. The effects on urinary corticosteroids ("11-oxysteroids") were variable, probably due to two factors: (1) suppression of the adrenal secretion by cortisone and (2) excretion of a portion of the cortisone in this fraction of the urine.

In all cases except one, daily doses of 25 mg. of cortisone decreased the output of 17-ketosteroids to as low a level (4 to 7 mg. daily) as did doses of 50 or 100 mg. per day, although the rapidity of the fall was somewhat slower with the smaller doses. The suppression was maintained with 25 mg. of cortisone daily, or 50 mg. q. 2 d., as long as treatment was continued, the longest period to date being six months. In small infants it is probable that doses as low as 10 or 15 mg. per day will suffice.

With the small doses of cortisone used no changes in carbohydrate metabolism were observed. There was some decrease of the excessive protein anabolism. Cortisone seemed to have slight sodium-retaining effect; but in cases in which there are Addisonian symptoms additional treatment with desoxycorticosterone will probably be necessary.

That cortisone actually suppressed the virilization characteristic of this disorder was suggested by the fact that there was decrease of acne and seborrhea in a number of cases, diminished rate of growth of axillary hair in one case and cessation of clitoral erections in another case. The two female pseudohermaphrodites treated longest have shown definite breast development and estrogenic changes of the vaginal smear, and one of these patients has now had five regular menstrual periods.

Dr. Fisher: Dr. Wilkins' paper is open for discussion.

Dr. Howard: The observations reported to us by Dr. Wilkins seem to me among the most exciting in a long time. They are of great therapeutic importance, but still more significant perhaps in their implications on physiological concepts. The disease pattern of congenital female pseudohermaphroditism is a very distinctive one; and so far as we are aware, it is only found associated with, and presumably due to, adrenal hyperplasia. Apparently during fetal development the adrenals go haywire and produce excessive quantities of hormones, perhaps normal hormones, perhaps abnormal. Administration of large doses of estrogens has altered the picture but little; some minor breast development and occasional spotty uterine bleeding.

However, here as the result of cortisone, the whole picture has been one of rapid reversal to the normal, even to the disappearance of the hypertension which we have felt was also in some way due to the adrenal abnormality. In a way it is like fighting fire with fire, for administration of cortisone, especially to children who seem more susceptible than adults, produces hypertension and some evidences of virilization. The principles involved in the response to cortisone in these cases seem worthy of some speculation. Dr. Wilkins has suggested that the cortisone may have acted as a tamponade to reduce a possible overactive pituitary ACTH production. This explanation may be correct, but it seems to me that the massive hormone production of the hyperplastic adrenals would probably have already served this purpose; and that the primary trouble may be in the adrenals themselves. The small dosage of cortisone required to initiate and maintain the altered physiology of the adrenals is particularly striking. It would seem as if here we were dealing with a totally different type of action of cortisone from the one where we give large doses to counteract the clinical manifestations of rheumatoid arthritis or diseases of hypersensitivity. In the latter, one visualizes a peripheral effect of the hormone which gives the cells a resistance to the irritating effects of agents which ordinarily annoy them excessively. It seems to me that in these pseudohermaphrodites the adrenals may have been affected directly by the cortisone, so that a system which produced abnormal hormones in excess reverted to normal hormone production. It looks as though a mixed up intermediary metabolism might have been provided with a constituent which altered the pathway and set the machinery right. It will be of great interest to see whether reversion to the original picture takes place when cortisone is stopped.

The natural next experimental step to Dr. Wilkins' pioneer work is to see if other abnormal adrenal states will also be affected along similar theoretical principles. A single patient with Cushing's syndrome has been given cortisone with striking improvement. The only trouble is that this patient had so many complications that at the time the cortisone was given, she could hardly have been called a representative case of Cushing's syndrome.

Dr. Fisher: Is there any other discussion?

Dr. Friedenwald: I would like to ask Dr. Wilkins whether he has produced lipemia in any of his patients.

Dr. Wilkins: No, we have not found any hyperlipemia in these cases and I think there have been several of the cases in which cholesterol has fallen with cortisone.

Dr. Fisher: Are there any other questions or discussion? If not, I would like to thank the speakers. The meeting is adjourned.

BOOKS RECEIVED FOR REVIEW

Bronchoesophagology. By CHEVALIER JACKSON and CHEVALIER L. JACKSON. 366 pp. $12.50. W. B. Saunders Co., Philadelphia, Pa.

The Care and Breeding of Laboratory Animals. Edited by EDMOND J. FARRIS. 515 pp. $8.00. John Wiley & Sons, Inc., New York.

Child Psychiatry in the Community. By HAROLD A. GREENBERG in collaboration with JULIAN H. PATHMAN, HELEN A. SUTTON, and MARJORIE M. BROWNE. 296 pp. $3.50 G. P. Putnam's Sons, Minton, Balch & Co., New York.

Color Atlas of Pathology. Prepared under the auspices of the U. S. Naval Medical School of the National Naval Medical Center, Bethesda, Md. 546 pp. $20.00. J. B. Lippincott Co., Philadelphia, Pa.

The Community and Public Health Nursing. By EDITH WENSLEY. 250 pp. $3.50. The Macmillan Co., New York.

Das Autogene Training (Konzentrative Selbstentspannung). By J. H. SCHULTZ. 325 pp. Ganzleinen DM 27. Georg Thieme Verlag, Stuttgart.

Differential Diagnosis of Internal Diseases. Clinical Analysis and Synthesis of Symptoms and Signs. By JULIUS BAUER. 866 pp. $12.00. Grune & Stratton, New York.

Einfuhrung in Die Hals-Nasen-Ohren Heilkunde. By PAUL FALK. 175 pp. Ganzleinen DM 19.80. Georg Thieme Verlag, Stuttgart.

Lehrbuch der Inneren Medisin (in 2 Banden). By HELMUT DENNIG. 1117 pp. Ganzleinen DM 39. Georg Thieme Verlag, Stuttgart.

Oral Pathology, 3rd edition. By KURT H. THOMA. 1592 pp. $17.50. The C. V. Mosby Co., St. Louis, Missouri.

Physical Examination in Health and Disease. By RUDOLPH H. KAMPMEIER. 821 pp. $8.00. The F. A. Davis Co., Philadelphia, Pa.

Regional Orthopedic Surgery. By PAUL C. COLONNA. 706 pp. $11.50. W. B. Saunders Co., Philadalphia, Pa.

Untersuchungstechnik des Chirurgen. By PAUL ROSTOCK. 330 pp. Ganzleinen DM 24. Georg Thieme Verlag, Stuttgart.